

TRADE SECRET

Study Title

Dimethyl Adipate (DMA): Static, Acute, 48-Hour EC₅₀ to
Daphnia magna

Laboratory Project ID: DuPont-11949

TEST GUIDELINES: U.S. EPA Office of Toxic Substances (1982)

OECD Guideline for Testing Chemicals
Section 2: Effects on Biotic Systems, Number 202 (1984)

Commission Directive 92/69/EEC
EEC Method C.2 (1992)

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STUDY COMPLETED ON: May 23, 2003

PERFORMING LABORATORY: E.I. du Pont de Nemours and Company
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WORK REQUEST NUMBER: 14398

STUDY CODE NUMBER: 241

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with U.S. EPA TSCA (40 CFR part 792) Good Laboratory Practice Standards, which are consistent with the OECD Principles of Good Laboratory Practice (as revised in 1997) published in ENV/MC/CHEM(98)17 and MAFF Japan Good Laboratory Practice Standards (59 NohSan No. 3850).

The test substance was characterized by the sponsor prior to the initiation of this study. Although the characterization was not performed under Good Laboratory Practice Standards, the accuracy of the data is considered sufficient for the purposes of this study.

Study Director: _____

Alan Samel, M.S.
Research Ecotoxicologist
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Date

QUALITY ASSURANCE STATEMENT

Haskell Sample Number(s):

24301

Dates of Inspections:

Protocol: January 17, 2003

Conduct: January 21, 2003

Records, Reports: April 10-11, 2003

Dates Findings Reported to:

Study Director: April 11, 2003

Management: April 25, 2003

Reported by:

Wonda K. Kelly
Quality Assurance Auditor

Date

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Analytical Report by: _____

Bogdan Szostek, Ph.D.
Senior Research Chemist

Date

Issued by Study Director: _____

Alan Samel, M.S.
Research Ecotoxicologist

Date

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STUDY INFORMATION

9th Collective Nomenclature: Hexanedioic acid, dimethyl ester

Synonyms/Codes:

- Dimethyl adipate
- DBE-6
- DMA
- Adipic acid, dimethyl ester
- Dimethyl hexanedioate
- Methyl adipate
- Dibasic ester-6
- J806339-A (Lot No.)

Haskell Number: 24301

CAS Registry Number: 627-93-0

Composition: 98.824% Dimethyl adipate (DMA-DBE6) by GC

Known Impurities: 0.607% Dimethyl glutarate (DMG-DBE5) by GC
4.8 ppm HCN

Physical Characteristics: Colorless liquid

Stability: The test substance was stable under the conditions of the study.

Sponsor: Dibasic Esters Group
1100 New York Avenue, N.W., Suite 1090
Washington, DC 20005

Study Initiated/Completed: January 16, 2003 / (see report cover page)

STUDY PERSONNEL

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SUMMARY

The acute toxicity of Dimethyl Adipate (DMA) to unfed *Daphnia magna* neonates, less than 24 hours old at test start, was determined in an unaerated, 48-hour, static test. The test was conducted in accordance with the Organisation for Economic Co-Operation and Development (OECD) Guideline for Testing Chemicals: 202; the European Economic Community 92/69 Annex V - Method C.2; and the United States Environmental Protection Agency, Office of Toxic Substances.

The study was conducted with 5 concentrations of DMA and a dilution water control at a mean temperature of 20.0°C (range of 19.9-20.2°C). Four replicates with 5 daphnids per replicate were used per test substance concentration and control.

Exposure of daphnids to mean, measured DMA concentrations of 6.9, 14, 29, 58, and 120 mg/L resulted in 0, 0, 0, 10, and 100% immobility, respectively, at the end of 48 hours. Mean, measured concentrations ranged from 92 to 100% of the nominal concentrations adjusted for 98.8% purity by analysis. No immobility or sublethal effects were observed in the dilution water control daphnids. The highest mean, measured concentration causing no immobility at test end was 29 mg/L. The lowest mean, measured concentration causing 100% immobility at test end was 120 mg/L. Mean, measured DMA concentrations were used for calculation of EC₅₀ values. The 48-hour EC₅₀, based on mean, measured concentrations of DMA and immobility, was 72 mg/L.

DMA exhibited medium concern for aquatic hazard⁽¹⁾ to unfed *Daphnia magna* in an unaerated 48-hour acute toxicity test.

The results are summarized as follows:

Nominal concentrations of DMA, mg/L ^a	dilution water control, 7.5, 15, 30, 60, and 120
Mean, measured concentrations of DMA, mg/L	ND, ^b 6.9, 14, 29, 58, and 120
48-hour EC ₅₀ (95% confidence interval) for DMA, based on mean, measured concentrations, mg/L	72 (61 to 88)

a Adjusted for 98.8% purity by analysis during preparation.

b ND denotes none detected at or above the limit of detection of 0.19 mg/L.

INTRODUCTION

The objective of this study was to assess the acute toxicity of Dimethyl Adipate (DMA) to unfed *Daphnia magna* neonates, less than 24 hours old at test start, during an unaerated, static, 48-hour test.

MATERIALS AND METHODS

A. Test Substance

The test substance, DMA, was supplied as a colorless liquid. The test substance contains 98.8% DMA by analysis.

The solubility of DMA in a solution prepared in Haskell Laboratory well water (HLWW) at 20°C was verified at 214 mg/L. The solubility of DMA in a solution prepared in HLWW at 10°C was verified at 215 mg/L.⁽²⁾

The stability of DMA in HLWW was demonstrated for 96 hours at approximately 20°C and 4°C.⁽²⁾

B. Test Solution Preparation

Test substance solutions were prepared by dilution from a stock solution of DMA in HLWW. Nominal concentrations of test substance solutions were not adjusted for purity. The stock solution of 120 mg/L was prepared by weighing out the appropriate amount of test material and adding it to 1000 mL HLWW in a 2L Erlenmeyer flask. The final volume was brought to 2L with HLWW and stirred for approximately 2 hours. Test solutions were prepared by adding the appropriate volume of the stock solution to a 1L beaker, bringing it to a final volume of 1L with HLWW and stirring for approximately 23 minutes. The stock solution was clear and colorless with no insoluble material present. Test substance solutions were clear and colorless with no insoluble material present.

C. Dilution Water

Dilution water originated from the Haskell Laboratory well which is 378-feet deep and is cased and sealed to bedrock. The hardness of the HLWW is adjusted to approximately 100-140 mg/L as CaCO₃ by the flow-proportioned addition of CaCl₂. The HLWW is then aerated, passed through a green sand filter to remove iron, and filtered through 50-, 10-, and 5-μm filters to remove particulates. The water is heated or chilled as appropriate and distributed through aged polyvinyl chloride piping. The dilution water is analyzed twice yearly for major anions and

cations, metals, total organochlorine and organophosphate pesticides, and polychlorinated biphenyls (Table 1). The dilution water meets OECD⁽³⁾ and ASTM⁽⁴⁾ specifications.

D. Test Organism Culture

Daphnia magna were reared at Haskell Laboratory in 1000-mL Pyrex[®] beakers (10 per beaker) which contained 1000 mL of aerated, filtered HLWW held at 20.5°C. Daphnids were fed on a daily basis with a yeast, cereal leaves and trout chow (YCT) mixture (standardized to 1700-2100 mg/L total solids) and the green alga, *Selenastrum capricornutum*, at a rate of 62,500 cells/mL of culture media. The combination of YCT and alga is equivalent to 0.1-0.2 mg total organic carbon per daphnid/day. Neonates used in this test were less than 24 hours old and were collected from the 7th and 10th brood of 20- and 28-day old parent daphnids, respectively. Sickness, injury, and abnormalities were not seen and ephippia were not being produced by the parent daphnids. No adult immobility was seen in the cultures used for testing during the 48-hour pretest period. *Daphnia magna* were identified by labels on the culture beakers and test chambers.

E. Test Methods^(3,5,6)

Five nominal concentrations and a dilution water control were used in this study. Nominal concentrations of DMA (not adjusted for 98.8% purity) of 7.5, 15, 30, 60, and 120 mg/L were chosen for the definitive test based on the results of a preliminary rangefinding study (see Results and Discussion). The dilution water control contained no solvent.

Pyrex[®] beakers (250-mL) containing 200 mL of test solution (6.8-cm test solution depth) were used as test chambers. Four replicate test chambers were used per test concentration with 5 daphnids in each chamber (20 daphnids per concentration). The test chambers were covered with a glass plate during the test. Random numbers were used to assign test concentrations to the test chambers and position of test concentrations in the water bath.

Daphnia magna neonates, less than 24 hours old, were used in this study. Daphnids were assigned to the test chambers using random numbers. Addition of daphnids to test solutions was initiated about 57 minutes after mixing of the test solutions was completed. Observations of test organisms were made daily. The criterion for the effect (immobility) was a lack of reaction to application of a gentle stimulus. Daphnids were not fed during the test.

A recirculating water bath was used to maintain mean temperature in the test chambers during the 48-hour test at approximately 20.0°C with a range of 19.9 to 20.2 °C (Table 6). In addition, a continuously-recording thermometer was used to check for temperature variation in the water bath. A photoperiod of 16 hours light (approximately 346 - 674 Lux) and 8 hours darkness was employed which included 30 minutes of transitional light (18 - 57 Lux) preceding and following the 16-hour light interval.

Dissolved oxygen concentration, pH, and temperature were measured in all replicates of the control and test substance concentrations. These measurements were taken before daphnids were

added at test start, and at test end or at total immobility in a concentration. Total alkalinity, EDTA hardness, and conductivity of the dilution water controls and highest test substance concentration were measured before daphnids were added at the beginning of the test. Test solutions were not aerated during the test and were disposed of in an appropriate manner at test end.

F. Sample Preparation and Chemical Analysis

1. Sample Collection and Treatment

One sample plus a back-up sample of each test solution was received from each test concentration including the control on day 0 of the test before the test solutions were poured into the replicate test chambers. One sample plus a back-up sample was also received from 2 of the 4 replicate chambers (C and D) at all test concentrations including the controls at test end (day 2).

Concentrations of DMA were measured directly by high performance liquid chromatography (HPLC) on the day of sample receipt. The 60 mg/L and 120 mg/L samples were diluted 1:2 and 1:4 with HLWW before analysis, respectively.

2. Instrument and Conditions

Instrument:	Hewlett Packard Model 1100 HPLC
Column:	Zorbax RX-C18 2.1 x 150 mm 5 micron
Mobile Phase:	70% Water 30% Acetonitrile
Flow Rate:	0.3 mL/min
Column Temperature:	35°C
Injection Volume:	100 µL
Detector:	UV absorbance at 210 nm
Run Time:	10 minutes

3. Quantitation

A stock solution of the reference standard, DMA (purity 98.8%), was made by dissolving approximately 10 mg of the standard in 10 mL of acetonitrile. Appropriate aliquots of the stock solution were diluted with dilution water (HLWW) to prepare calibration standard solutions with concentrations ranging from 5.1 to 51 mg/L. Duplicate injections of test and calibration standard solutions were made and peak heights were determined electronically.

The calibration standard curve was generated by regression analysis of the peak height data from chromatography of the calibration standard solutions. Data for test solutions were compared to the calibration standard curve to determine concentrations of DMA. The limit of detection (LOD) and limit of quantitation (LOQ) were determined by calculating the average noise level in chromatograms of the water control solution and comparing them to the signal of a calibration standard of known concentration. Two chromatograms were examined for noise-related peaks near the retention time of the analyte. The LOD was calculated as 3 times the concentration

equivalent of the mean noise level. The LOQ was calculated as 10 times the concentration equivalent of the mean noise level.

G. Statistical Analysis

The 24-hour EC₅₀ value was calculated by probit analysis⁽⁷⁾ based on mean, measured DMA concentrations. The 48-hour EC₅₀ value was calculated by the moving average-angle method⁽⁸⁾ based on mean, measured DMA concentrations. The highest mean, measured concentration causing no immobility at test end and the lowest mean, measured concentration causing 100% immobility at test end were assessed by visual observation.

RESULTS AND DISCUSSION

A. Analytical Report

1. Chromatographic Results

DMA eluted as a well-resolved peak with a retention time of approximately 4.4 minutes. A typical calibration standard curve is shown in Figure 1. Representative chromatograms of a calibration standard solution, a dilution water control solution sample, and a test solution sample are presented in Figures 2 to 4, respectively.

The LOD and LOQ were determined to be 0.19 mg/L and 0.63 mg/L, respectively.

2. Test Solution Results

Mean, measured values of DMA ranged from 92 to 100% of the targeted nominal concentrations not adjusted for test substance purity of 98.8% (Table 2). All measured values of DMA were within 1.5X of the lowest value for all samples within a concentration. These data indicate that DMA concentrations were maintained at acceptable levels throughout the definitive test.

Control solutions showed no detectable concentrations of DMA (Table 2).

B. In-Life Report

1. Rangefinding Study

A static rangefinding study with 10 daphnids per replicate, one replicate per concentration, was conducted using a dilution water control and nominal concentrations of 0.1, 1.0, 10, and 120 mg/L. At the end of 48 hours, the respective immobility values were 0, 0, 10, 0, and 80%. Test substance solutions were clear and colorless with no undissolved test substance.

2. Definitive Study

Nominal concentrations (not adjusted for 98.8% purity during preparation) for the definitive study were 7.5, 15, 30, 60, and 120 mg/L (5 daphnids per replicate, four replicates per concentration). A dilution water control was used in this study. Mean, measured concentrations were 6.9, 14, 29, 58, and 120 mg/L, respectively. Control solutions showed no detectable concentrations of DMA. All test substance solutions were clear and colorless with no undissolved test substance.

Dilution water quality was acceptable based on OECD⁽³⁾ and ASTM⁽⁴⁾ dilution water criteria. Based on the most recent semi-annual dilution water analysis (Table 1), contaminant concentrations were below concentrations that could be expected to affect the integrity of a study. All chemical and physical parameters for the definitive test (Tables 3 - 6) were within expected

ranges. Total alkalinity, EDTA hardness, and conductivity of the dilution water control and highest test substance concentration at test start ranged from 44 to 49 mg/L CaCO₃, 120 to 124 mg/L CaCO₃, and 285 to 300 µmhos/cm, respectively. During the test, dissolved oxygen concentrations ranged from 8.3 to 9.0 mg/L, pH ranged from 7.5 to 8.0, and mean temperature was 20.0°C with a range of 19.9 to 20.2°C.

Data on daily immobility and sublethal effects are presented in Tables 7 and 8, respectively. Exposure of daphnids to mean, measured DMA concentrations of 6.9, 14, 29, 58, and 120 mg/L resulted in 0, 0, 0, 10, and 100% immobility, respectively, at the end of the 48 hours. No immobility or sublethal effects were observed in the dilution water control or the mean, measured concentration of 6.9 mg/L. Floating daphnids (2 out of 20) were observed in the 14 mg/L mean, measured test concentration at the end of the study. In the 29 mg/L mean, measured test concentration lethargic daphnids (2 out of 20) were observed at the end of the study. In the 58 mg/L mean, measured test concentration, lethargic daphnids (15 out of 18) and lethargic and pale daphnids (2 out of 18) were observed at the end of the study.

A summary of the EC₅₀ values and 95% confidence intervals at specific time intervals is presented in Table 9. Mean, measured concentrations of DMA versus 48-hour immobility data are graphically represented in Figure 5. The 24-hour EC₅₀, based on mean, measured concentrations of DMA, was 119 mg/L with 95% fiducial limits of 103 to 148 mg/L. The 48-hour EC₅₀, based on mean, measured concentration of DMA, was 72 mg/L with 95% confidence limits of 61 to 88 mg/L. The highest mean, measured concentration causing no immobility at test end was 29 mg/L. The lowest mean, measured concentration causing 100% immobility at test end was 120 mg/L.

CONCLUSION

DMA was assessed for acute toxicity to unfed *Daphnia magna* neonates, less than 24 hours old, in an unaerated, static, 48-hour test. The 48-hour EC₅₀, based on mean, measured concentrations of DMA and immobility, was 72 mg/L.

RECORDS AND SAMPLE STORAGE

All data and records for analytical characterizations conducted by the sponsor will be archived by the sponsor. Laboratory-specific or site-specific raw data, such as personnel files and equipment records will be retained by the facility where the work was done.

Specimens (if applicable), raw data, and the final report will be retained at Haskell Laboratory, Newark, Delaware, or at Iron Mountain Records Management, Wilmington, Delaware (formerly known as the E.I. du Pont de Nemours and Company Records Management Center).

REFERENCES

1. Smrchek, J., Clements, R., Morcock, R., and Rabert, W. (1993). Assessing ecological hazard under TSCA: methods and evaluation of data. Environmental Toxicology and Risk Assessment, ASTM STP 1179. (W.G. Landis, J.S. Hughes, and M.A. Lewis, Eds.), pp. 22-39. American Society for Testing and Materials, Philadelphia.
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4. American Society for Testing and Materials (ASTM). (1988). Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians. E 729-88a. Annual Book of ASTM Standards, Vol. 11.04.
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7. SAS[®] User's Guide, *The PROBIT Procedure*, SAS Institute Inc., Cary, North Carolina. pp. 639 - 646.
8. Peltier, W.H. and Weber, C.I., Eds. (1985). *Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms*. EPA/600/4-85-013. United States Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio.

TABLES

TABLE 1
CHEMICAL CHARACTERISTICS OF HASKELL LABORATORY WELL WATER^a

Parameter	MDL ^b	Analytical Value	Parameter	MDL ^b	Analytical Value
BOD, mg/L	3.6	ND ^c	Lead, mg/L	0.0089	ND
COD, mg/L	1.7	ND	Magnesium, mg/L	0.0195	3.92
DOC, mg/L	0.6	0.55 ^d	Manganese, mg/L	0.0005	0.0037 J ^e
TOC, mg/L	0.5	0.691 J	MBAS/LAS, mg/L	0.035	ND
Total Kjeldahl N, mg/L	0.3	ND	Mercury, mg/L	0.000079	ND
Ammonia N, mg/L	0.02	0.036 J	Nickel, mg/L	0.0019	ND
Turbidity, NTU	0.059	0.096 J	Nitrite, mg/L	0.015	ND
Phenolics, mg/L	0.012	ND	Nitrate, mg/L	0.04	ND
Color, apparent Co/Pt ^f	5.0	ND	Ortho-phosphate, mg/L	0.0066	ND
Solids			Potassium, mg/L	0.0472	2.56
total suspended, mg/L	1.5	ND	Selenium, mg/L	0.0011	ND
Aluminum, mg/L	0.0477	ND	Silver, mg/L	0.00005	ND
Antimony, mg/L	0.0099	ND	Sodium, mg/L	0.25	8.25
Arsenic, mg/L	0.0014	ND	Sulfate, mg/L	1.5	8.0
Beryllium, mg/L	0.0005	ND	Sulfide, mg/L	0.022	ND
Boron, mg/L	0.0088	ND	Zinc, mg/L	0.0049	ND
Bromide, mg/L	2.0	ND	Ca/Mg	NA ^g	10.41
Cadmium, mg/L	0.00008	ND	Na/K	NA	3.22
Calcium, mg/L	0.0493	40.8	Volatile priority		
Chloride, mg/L	6.0	56.1	pollutants, µg/L	0.5-40	ND
Chlorine, residual, mg/L	0.04	ND	Acid extractable		
Chromium, mg/L	0.00092	ND	priority pollutants, µg/L	1.0-19	ND
Cobalt, mg/L	0.0017	ND	Base/neutral		
Copper, mg/L	0.0016	0.0043 J	priority pollutants, µg/L	1.0-19	ND
Cyanide, mg/L	0.004	ND	Pesticides/PCBs, µg/L	0.0019-0.29	ND
Iron, mg/L	0.0349	ND	Organophosphate		
Fluoride, mg/L	0.4	0.56	pesticides, µg/L	0.38-0.57	ND

^a Sample analyses performed at Lancaster Laboratories, Lancaster, Pennsylvania, date of sample collection 06 November 2002 unless indicated otherwise,

^b MDL = method detection limit, ^c ND indicates not detected at the MDL, ^d Value corrected for the filter blank, ^e A "J" follows analytical values which were greater than the MDL but less than the limit of quantitation, ^f Units based on cobalt/platinum reference, ^g NA = not applicable.

TABLE 2
MEASURED CONCENTRATIONS OF DMA IN TEST SOLUTIONS

Nominal DMA Concentration (mg/L)	Nominal DMA Concentration, adjusted for purity (mg/L) ^a	Measured DMA Concentration (mg/L)		Mean, Measured Concentration (mg/L) ^d	Percent Recovery (%) ^e
		Day 0 ^b	Day 2 ^c		
Water Control C	0.0	ND ^f	ND	---	---
Water Control D	0.0	ND	ND		
7.5 C	7.4	7.3	6.4	6.9	92
7.5 D	7.4		6.4		
15 C	15	15	13	14	93
15 D	15		13		
30 C	30	30	27	29	97
30 D	30		27		
60 C	60	59	56	58	97
60 D	60		56		
120 C	120	120	120	120	100
120 D	120		120		

a Nominal DMA concentration based on 98.8% purity by analysis.

b One sample of freshly prepared test solution was taken from each concentration level on day 0 before the test solutions were poured into the replicate test chambers.

c Two of 4 replicate chambers were sampled on day 2.

d Mean, measured DMA concentration was calculated as (Day 0 + (Day 2_C+Day 2_D)/2)/2

e Based on nominal concentration.

f ND denotes not detected. The limit of detection for DMA was 0.19 mg/L.

TABLE 3

WATER CHEMISTRY OF THE DILUTION WATER CONTROL AND HIGHEST TEST
SUBSTANCE CONCENTRATION AT TEST START

Mean, Measured DMA Concentration (mg/L)	Total Alkalinity (mg/L as CaCO ₃)	EDTA Hardness (mg/L as CaCO ₃)	Conductivity (µmhos/cm)
Water Control	49	124	285
120 mg/L	44	120	300

TABLE 4

DISSOLVED OXYGEN CONCENTRATION (mg/L)^Ψ OF DMA TEST SOLUTIONS

Mean, Measured DMA Concentration (mg/L)	0 Hours				48 Hours			
	A [†]	B [†]	C [†]	D [†]	A [†]	B [†]	C [†]	D [†]
Water Control	8.6	8.6	8.7	8.6	8.8	8.9	8.9	8.9
6.9	8.6	8.7	8.7	8.7	8.9	8.9	8.9	9.0
14	8.7	8.7	8.7	8.7	8.9	8.9	8.9	9.0
29	8.6	8.6	8.6	8.6	8.9	8.9	8.9	8.9
58	8.6	8.6	8.6	8.6	8.9	9.0	8.9	9.0
120	8.3	8.3	8.4	8.4	9.0	8.9	8.9	8.9

Ψ The theoretical dissolved oxygen concentration at 100% saturation is 9.1 mg/L at 20.0°C.

† Replicate test chambers contained 5 daphnids each (total 20 daphnids per concentration) at test start.

TABLE 5
pH OF DMA TEST SOLUTIONS

Mean, Measured DMA Concentration (mg/L)	0 Hours				48 Hours			
	A [†]	B [†]	C [†]	D [†]	A [†]	B [†]	C [†]	D [†]
Water Control	7.5	7.5	7.5	7.5	8.0	8.0	8.0	8.0
6.9	7.6	7.6	7.6	7.6	8.0	8.0	8.0	8.0
14	7.6	7.6	7.6	7.6	8.0	8.0	8.0	8.0
29	7.6	7.6	7.6	7.6	8.0	8.0	8.0	8.0
58	7.6	7.6	7.6	7.6	8.0	7.9	7.9	7.9
120	7.7	7.7	7.7	7.7	7.9	7.9	7.9	7.9

† Replicate test chambers contained 5 daphnids each (total 20 daphnids per concentration) at test start.

TABLE 6
TEMPERATURE (°C) OF DMA TEST SOLUTIONS

Mean, Measured DMA Concentration (mg/L)	0 Hours				48 Hours			
	A [†]	B [†]	C [†]	D [†]	A [†]	B [†]	C [†]	D [†]
Water Control	20.1	20.1	20.1	20.1	20.0	20.0	19.9	19.9
6.9	20.0	20.1	20.1	20.1	19.9	19.9	19.9	19.9
14	20.1	20.1	20.1	20.1	19.9	19.9	19.9	19.9
29	20.1	20.2	20.2	20.1	19.9	19.9	19.9	19.9
58	20.1	20.1	20.2	20.1	19.9	19.9	19.9	19.9
120	20.2	20.2	20.1	20.1	19.9	19.9	19.9	19.9

† Replicate test chambers contained 5 daphnids each (total 20 daphnids per concentration) at test start.

TABLE 7

IMMOBILITY OF *Daphnia magna* AT 24 AND 48 HOURS IN AN UNAERATED, STATIC,
ACUTE TEST WITH DMA

Mean, Measured DMA Concentration (mg/L)	Number Immobile / Number at Test Start							
	24 Hours				48 Hours			
	A [†]	B [†]	C [†]	D [†]	A [†]	B [†]	C [†]	D [†]
Water Control	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
6.9	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
14	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
29	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
58	0/5	0/5	1/5	0/5	0/5	0/5	1/5	1/5
120	2/5	3/5	2/5	3/5	5/5	5/5	5/5	5/5

† Replicate test chambers contained 5 daphnids each (total 20 daphnids per concentration) at test start.

TABLE 8

SUBLETHAL EFFECTS IN *Daphnia magna* AT 24 AND 48 HOURS IN AN UNAERATED, STATIC, ACUTE TEST WITH DMA

Mean, Measured DMA Concentration (mg/L)	Number Affected / Number Alive							
	24 Hours				48 Hours			
	A [†]	B [†]	C [†]	D [†]	A [†]	B [†]	C [†]	D [†]
Water Control	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
6.9	0/5	1 ^d /5	0/5	1 ^d /5	0/5	0/5	0/5	0/5
14	0/5	1 ^a /5	0/5	1 ^d /5	0/5	0/5	1 ^d /5	1 ^d /5
29	0/5	0/5	0/5	1 ^a /5	1 ^a /5	0/5	1 ^a /5	0/5
58	3 ^a /5	5 ^a /5	4 ^a /4	5 ^a /5	2 ^a ,2 ^{ac} /5	5 ^a /5	4 ^a /4	4 ^a /4
120	3 ^a /3	2 ^a /2	3 ^a /3	2 ^a /2	I	I	I	I

† Replicate test chambers contained 5 daphnids each (total 20 daphnids per concentration) at test start.

OBSERVATION KEY

- | | |
|---------------------------------|---|
| a Daphnid lethargic | d Daphnid floating at surface |
| b Daphnid visibly small in size | e Daphnid accidentally crushed by pipette during transfer |
| c Daphnid pale in color | I Total immobility |

TABLE 9

MEAN, MEASURED CONCENTRATIONS OF DMA CAUSING IMMOBILITY TO 50%
(EC₅₀) OF *Daphnia magna* AT SPECIFIC TIME INTERVALS

Time	EC ₅₀ (mg/L) ^a	95% Confidence/Fiducial Intervals (mg/L) ^a		Slope ^a	Y-intercept ^a
		Lower	Upper		
24 Hours	119	103	148	0.03	-3.48
48 Hours	72	61	88	- ^b	-

- a 24-Hour values (including fiducial intervals) were calculated by probit analysis using untransformed data.
48-Hour values (including confidence intervals) calculated by the moving average-angle method.
- b Slope and y-intercept are not calculated by the moving average-angle method.

FIGURES

FIGURE 1

REPRESENTATIVE ANALYTICAL CALIBRATION STANDARD CURVE FOR DMA

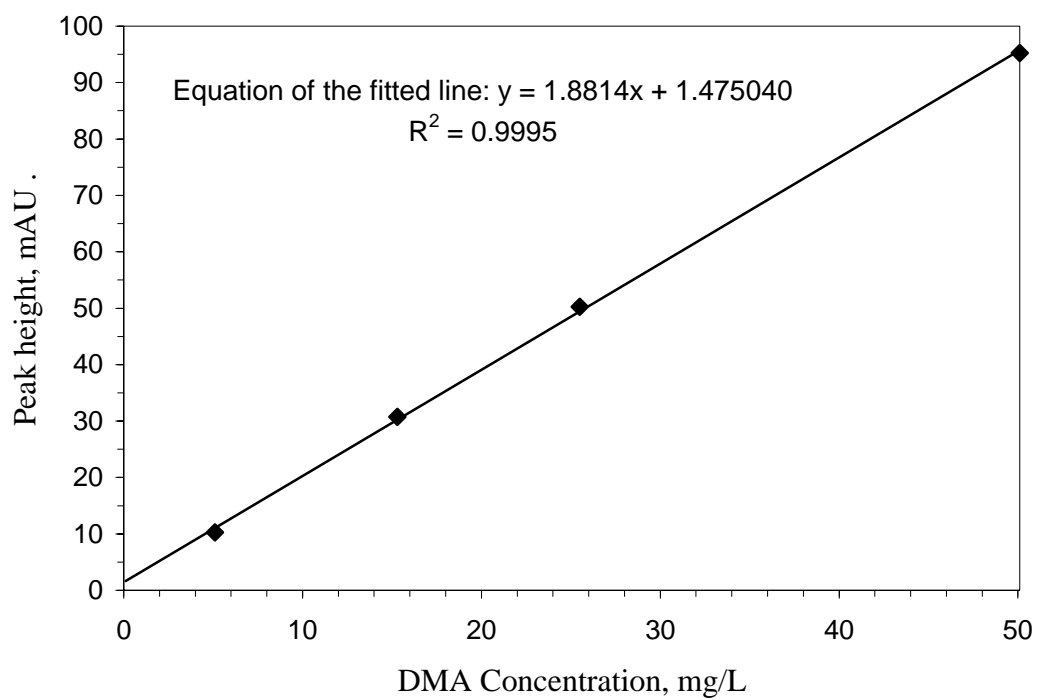
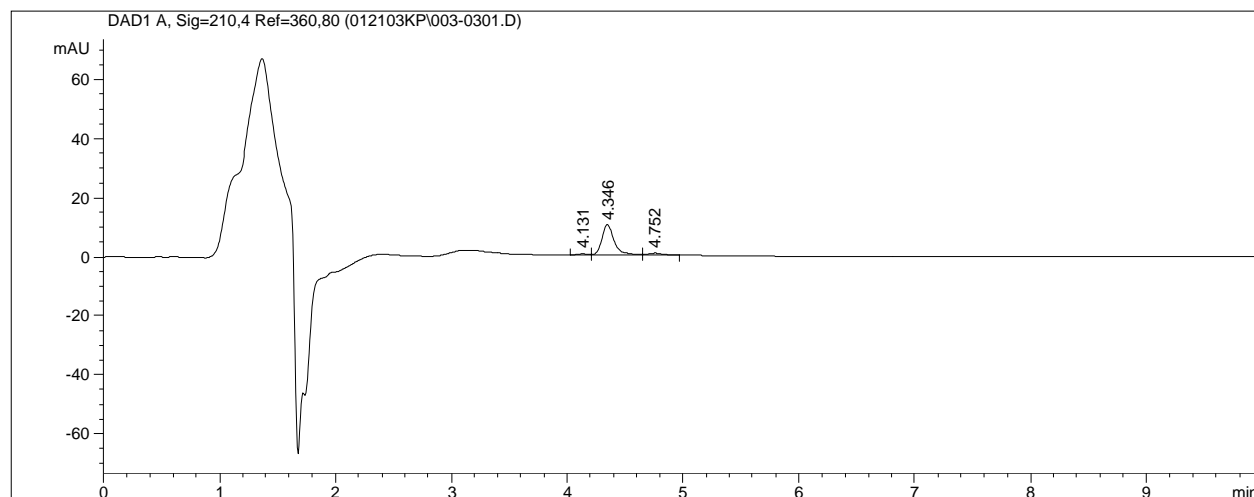


FIGURE 2

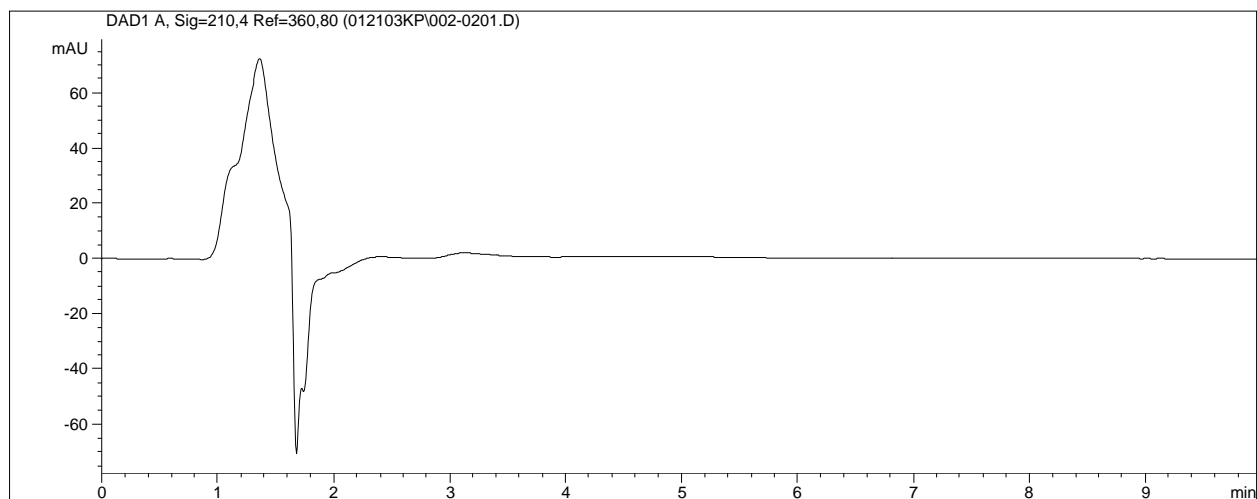
REPRESENTATIVE HPLC CHROMATOGRAM OF A
CALIBRATION STANDARD SOLUTION



DMA elutes at a retention time of approximately 4.4 minutes. Calibration standard solution contains DMA at a concentration of 5.1 mg/L.

FIGURE 3

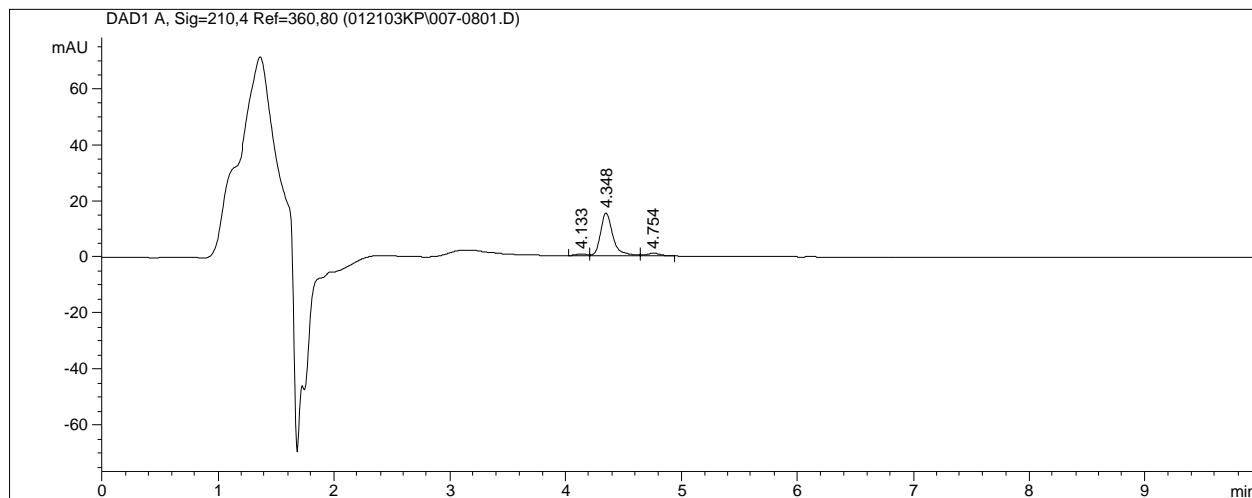
REPRESENTATIVE HPLC CHROMATOGRAM OF A
DILUTION WATER CONTROL SOLUTION



DMA would elute at a retention time of approximately 4.4 minutes.

FIGURE 4

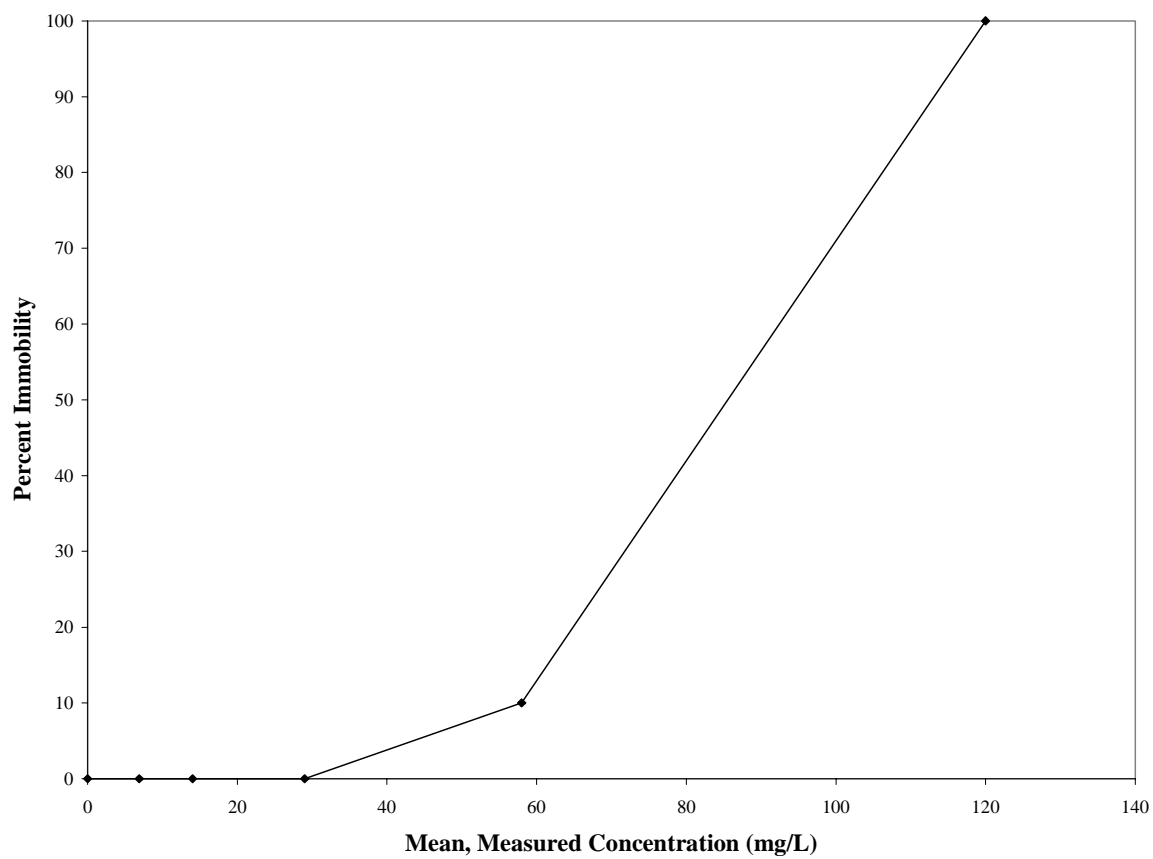
REPRESENTATIVE HPLC CHROMATOGRAM OF A DMA TEST SOLUTION



DMA elutes at a retention time of approximately 4.4 minutes. Test solution sample contains DMA at a nominal concentration of 7.5 mg/L.

FIGURE 5

MEAN, MEASURED CONCENTRATIONS OF DMA VERSUS
IMMOBILITY AT 48 HOURS



TRADE SECRET

Study Title

Dimethyl Adipate (DMA): Influence on Growth and Growth Rate
of the Green Alga *Selenastrum capricornutum*

Laboratory Project ID: DuPont-11950

TEST GUIDELINES: OECD Guideline for Testing Chemicals
Section 2: Effects on Biotic Systems, Number 201 (1984)

Commission Directive 92/69/EEC
EEC Method C3 (1992)

AUTHOR: Terry Lee Sloman, B.S.

STUDY COMPLETED ON: May 22, 2003

PERFORMING LABORATORY: E.I. du Pont de Nemours and Company
Haskell Laboratory for Health and Environmental Sciences
Elkton Road, P.O. Box 50
Newark, Delaware 19714-0050

WORK REQUEST NUMBER: 14398


SERVICE CODE NUMBER: 280

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with U.S. EPA TSCA (40 CFR part 792) Good Laboratory Practice Standards, which are consistent with the OECD Principles of Good Laboratory Practice (as revised in 1997) published in ENV/MC/CHEM(98)17 and MAFF Japan Good Laboratory Practice Standards (59 NohSan Number 3850).

The test substance was characterized by the sponsor prior to the initiation of this study. Although the characterization was not performed under Good Laboratory Practice Standards, the accuracy of the data is considered sufficient for the purposes of this study.

Study Director: _____

 Terry Lee Sloman, B.S.
Associate Scientist
E.I. du Pont de Nemours and Company

_____ Date

QUALITY ASSURANCE STATEMENT

Haskell Sample Number(s):

24301

Dates of Inspections:

Protocol: February 4, 2003

Conduct: February 4, 2003

Records, Reports: April 23,24, 2003

Dates Findings Reported to:

Study Director: April 24, 2003

Management: May 1, 2003

Reported by:

Robert C. Rhea, B.S., RQAP - GLP
Staff Quality Assurance Auditor

Date

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Analytical Reported by: _____
Bogdan Szostek, Ph.D.
Senior Research Chemist
Date

Statistical Reported by: _____
John W. Green, Ph.D., Ph.D.
Principal Research Statistician
Date

Issued by Study Director: _____
Terry Lee Sloman, B.S.
Associate Scientist
Date

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STUDY INFORMATION

9th Collective Nomenclature: Hexanedioic acid, dimethyl ester

Synonyms/Codes:

- Dimethyl adipate
- DBE-6
- DMA
- Adipic acid, dimethyl ester
- Dimethyl hexanedioate
- Methyl adipate
- Dibasic ester-6
- J806339-A (Lot No.)

Haskell Number: 24301

CAS Registry Number: 627-93-0

Composition: 98.824% Dimethyl adipate (DMA-DBE6) by GC

Known Impurities: 0.607% Dimethyl glutarate (DMG-DBE5) by GC
4.8 ppm HCN

Physical Characteristics: Colorless liquid

Stability: The test substance was stable under the conditions of the study based on analyses of the abiotic control.

Sponsor: Dibasic Esters Group
1100 New York Avenue, N.W., Suite 1090
Washington, DC 20005

Study Initiated/Completed: January 23, 2003 / (see report cover page)

STUDY PERSONNEL

Study Director: Terry Lee Sloman, B.S.

Management: Robert A. Hoke, Ph.D.

Analytical Associate: Keith B. Prickett, B.S.

Analytical Chemist: Bogdan Szostek, Ph.D.

Management: S. Mark Kennedy, Ph.D.

Statistical Analysis: John W. Green, Ph.D., Ph.D.

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Ecotoxicology Report Preparation: Wanda F. Dinbokowitz

Management: Nancy S. Selzer, M.S.

SUMMARY

A study was conducted to determine the effect of Dimethyl Adipate (DMA) on the growth and growth rate of the green alga *Selenastrum capricornutum*.^a The algae were exposed to nominal concentrations of 6.25, 12.5, 25, 50, and 100 mg DMA/liter of nutrient medium (ppm).

For the definitive (dose-response) test, the organisms were exposed for 72 hours (3 days) without test medium renewal. The effect was expressed as percent inhibition in growth based on healthy cell count (cell density), area under the growth curve, and growth rate relative to the blank (normal culture medium) control for the 72-hour (day 3) interval of the test.

The 72-hour results,^b based on nominal concentrations are as follows:

Healthy Cell Count

EC ₅₀	> 100 mg DMA/L
NOEC	12.5 mg DMA/L

Area Under the Growth Curve

EC ₅₀	> 100 mg DMA/L
NOEC	50 mg DMA/L

Growth Rate

EC ₅₀	> 100 mg DMA/L
NOEC	12.5 mg DMA/L

None of the definitive test concentrations exhibited a 50% or greater growth inhibition based on healthy cell count relative to the blank control. Therefore, a recovery test was not performed.

a Also known as *Pseudokirchneriella subcapitata*.

b The EC₅₀ is defined as the "effective concentration" producing a 50% inhibition of growth relative to the control. The NOEC is defined as the highest concentration of test substance that has no significant effect on the measured parameter relative to the control.

INTRODUCTION

This study was conducted to determine the effect of Dimethyl Adipate (DMA) on the growth and growth rate of the green alga *Selenastrum capricornutum*.^a

MATERIALS AND METHODS

A. Test Guidelines

The study design complies with the following test guidelines:

- Organisation for Economic Co-Operation and Development (OECD) (1984). 201 Effects on Biotic Systems. *Guideline for Testing of Chemicals*.
- European Economic Communities (EEC) (1992). Directive 92/69/EEC Annex V, Part C.3, Algal Inhibition Test. *Methods for the Determination of Toxicity*.

B. Test Substance

The test substance, DMA, was supplied by the sponsor as a colorless liquid.

The solubility of DMA in synthetic algal-assay-procedure (AAP) nutrient medium was verified at 215 mg/L at 25°C.⁽¹⁾

C. Test System

Selenastrum capricornutum, a freshwater, unicellular, non-motile, green alga, was used in this study.

1. Original Culture Source

The original culture source was the Department of Botany - Culture Collection of Algae - The University of Texas at Austin - Austin, Texas 78713-7640.

2. Culture Maintenance

The culture method for *Selenastrum capricornutum* was based on published literature.⁽²⁻³⁾ Prior to the study, *Selenastrum capricornutum* cultures were maintained under the same environmental conditions used in the study. The organisms were cultured in sterilized 250 mL Erlenmeyer flasks containing approximately 50 mL of filtered (= filter-sterilized) AAP nutrient medium and

^a Also known as *Pseudokirchneriella subcapitata*.

were aseptically transferred to fresh medium every 3 to 7 days. The flasks were fitted with foam stoppers to permit gas exchange.

D. Test Design

The test design used for the *Selenastrum capricornutum* definitive and recovery tests is described below:

Organism	Nutrient Medium	Flask Volume (mL)	Solution Volume (mL)	Volume Ratio
<i>Selenastrum capricornutum</i>	AAP	250	50	5:1

E. Test Preparation (Appendix A)

1. AAP Nutrient Medium Preparation for *Selenastrum capricornutum*

AAP nutrient medium⁽²⁾ was prepared by adding 1 mL of each of the 6 macronutrient stock solutions and 1 mL of the micronutrient stock solution to approximately 800 mL of Milli-Q[®] (deionized) water, with mixing after each addition. The volume of the medium was brought to 1 liter with additional Milli-Q[®] water. Appropriate proportions (1 mL of each stock solution for each 1 liter of medium) were used to prepare the larger volumes of the medium required for the definitive and recovery tests.

The medium pH was adjusted to 7.51 with 0.1N sodium hydroxide. For both the definitive and recovery tests, the medium was filter-sterilized using Corning[®] pre-sterilized filtration systems each with a 0.22 μ m cellulose acetate filter. The containers with the resulting filter-sterilized AAP nutrient medium for use in the definitive and recovery tests were stored in the refrigerator in the dark at approximately 4°C and acclimated to room temperature prior to use. Any remaining unused medium was properly stored.

2. Test Solution Preparation

Prior to the initiation of the definitive test, method development was conducted to determine the appropriate concentrations to be used in the definitive test.

A working stock solution was prepared by dissolving the test substance in 1000 mL filter-sterilized AAP nutrient medium for a nominal concentration of 100 mg DMA/L (= 100 ppm). Aliquots of the filter-sterilized AAP nutrient medium were used for the blank (normal culture medium) control solution. Test solutions were prepared using aliquots of the nominal 100 mg/L working stock solution and diluting with filter-sterilized AAP nutrient medium to make nominal concentrations of 6.25, 12.5, 25, and 50 mg DMA/L. Aliquots of the nominal 100 mg/L working stock solution were used for the 100 mg DMA/L test concentration solution and the abiotic (stability) control solution.

3. Test Culture Preparation

For each definitive control and test solution, 3 aliquots (50 mL each) were placed in separate sterilized 250 mL Erlenmeyer flasks fitted with sterilized foam stoppers. Each flask, excluding the abiotic control, was randomly assigned a number to eliminate bias while counting. To achieve the desired nominal concentration of approximately 10,000 *Selenastrum capricornutum* cells/mL at test initiation, an approximate 0.625 mL (= 625 μ L) aliquot of algal inoculum from a logarithmically growing stock culture was aseptically transferred to each flask, except the abiotic control.

F. Experimental Design

1. Definitive Test

The organisms were exposed for 72 hours (3 days), without test medium renewal. Test flasks were arranged on a lighted shelf in an environmental chamber in a non-systematic design and were re-positioned each working day. Each blank control, test concentration, and abiotic control was tested as 3 replicates. Cell counts were made approximately 24, 48, and 72 hours after the definitive test initiation (0-hour or day 0).

2. Recovery Test

Since none of the definitive test concentrations exhibited a 50% or greater growth inhibition based on healthy cell count relative to the blank control, a recovery test was not necessary.

G. Test Solutions

Solutions used in the definitive test were as follows:

Blank Control AAP nutrient medium containing no DMA

Treatment nominal 6.25, 12.5, 25, 50, and 100 mg DMA/liter nutrient medium

Abiotic Control nominal 100 mg DMA/liter nutrient medium; (no
Selenastrum capricornutum)

H. Test Conditions - Definitive and Recovery Tests

The control and test flasks were placed in a chamber (Hotpack model 06084) and air temperature in the chamber was recorded continuously with a continuous temperature recorder (Dickson Model SL445C7). The algae were incubated for 72 hours without test medium renewal for the definitive test. Illumination was supplied by cool-white fluorescent tubes. The **target** environmental parameters are described in the following table.

Test	Initial Population	Illumination (lumens/m ² = lux)	Photo- Period (hours)	Shaking Speed (rpms)	Temperature (°C)
Definitive	10,000 cells/mL	6000 to 10,000	24	100	24 ± 2
Recovery	variable; based on definitive test, termination-day counts from selected test concentrations	6000 to 10,000	24	100	24 ± 2

I. *Selenastrum capricornutum* Growth Measurement

1. Definitive Test

Selenastrum capricornutum growth measurement was determined by visually counting the number of cells taken from an approximate 0.2 mL sample from each flask at approximately 24, 48, and 72 hours from the definitive test initiation. The counts were conducted using a hemacytometer and a compound microscope. An aliquot of each sample was loaded into the hemacytometer and 16 grids were selected. All cells located in the 16 grids were counted and recorded as healthy or unhealthy. The total number of cells counted was multiplied by 10,000 to determine the number of cells per milliliter. Cells outside these 16 grids were not counted nor included in the total number. Observations and counts of healthy and unhealthy cells (e.g., deformed, senescent, stunted) were recorded in the study records. Counts were made at approximately the same time each day. Statistical calculations of the EC₅₀ and NOEC were based on mean healthy cell counts and nominal concentrations.

J. Statistical Analyses

The mean healthy cell count, area under the growth curve, and growth rate at the 72-hour interval for each test concentration were expressed relative to the blank control. All calculations were based on the nominal concentrations.

The area under the growth curves for each interval were calculated according to the following formula:

$$A = [0.5 \times t_1 \times (N_1 - N_0)] + [0.5 \times (t_2 - t_1) \times (N_2 + N_1 - 2N_0)] \\ + \dots + [0.5 \times (t_n - t_{n-1}) \times (N_n + N_{n-1} - 2N_0)]$$

where t_1 , t_2 , t_{n-1} , and t_n are times of observation measured from the initiation of the test and N_0 , N_1 , N_{n-1} , and N_n are the initial and subsequent healthy cell counts (cells/mL) corresponding to the observation times.

The growth rate (μ) for each test concentration and for the controls were calculated according to the following formula:

$$\mu = \frac{\ln(N_n / N_0)}{t_n}$$

where t_n is the time of observation measured from the initiation of the test and N_0 and N_n are the initial and subsequent healthy cell counts (cells/mL) corresponding to the observation time.

The percent growth inhibition, % I, was calculated according to the following formula:

$$\% I = \frac{C - T}{C} \times 100$$

where C is equal to the mean healthy cell count, area under the growth curve, or growth rate for the blank control at a selected sampling interval and T is the mean healthy cell count, area under the growth curve, or growth rate for each test concentration at a selected sampling interval. Negative values of inhibition indicate stimulation of growth.

The means and standard errors of the healthy cell counts, areas under the growth curve, and growth rate for each test concentration and the blank control were calculated using standard procedures.⁽⁴⁾

The healthy cell counts, areas under the growth curve, and growth rates were used to calculate the EC₅₀ value. This “effective concentration” was defined as the concentration producing a 50% inhibition of growth relative to the blank control. The EC₅₀ value and associated 95% confidence limits were determined by weighted least-squares non-linear regression of the log of the test concentration against the measured parameter. The NOEC, defined as the highest concentration of test substance that had no significant effect on the measured parameter relative to the blank control, was determined from a trend test (Jonckheere-Terpstra) applied in a step-down manner. All tests of significance were at $\alpha = 0.05$.⁽⁵⁾

K. Analysis of Test Solutions - Definitive Test

1. Sample Collection

The concentrations of DMA in the working stock (nominal 100 mg DMA/L), blank control, and test concentration (nominal 6.25, 12.5, 25, and 50 mg DMA/L) solutions were verified at test initiation (day 0). An aliquot from each of the blank control and test concentration solutions was taken prior to the addition of *Selenastrum capricornutum*. The pH was determined for each of the primary stock, blank control, and test concentration solutions.

The concentrations of DMA in the blank control, test concentration (nominal 6.25, 12.5, 25, 50, and 100 mg DMA/L), and abiotic control (nominal 100 mg/L DMA/L) solutions were verified at test termination (day 3). These samples were prepared by pooling all 3 replicates for each of the

control and test concentration solutions and taking an aliquot from each of the pooled samples. The pH was determined for each of these day 3 samples.

The concentrations of in each of the test solutions were determined by high performance liquid chromatography (HPLC).

2. Sample Treatment

An aliquot from the blank control, working stock, and test concentrations were sampled at the study start (day 0) and submitted for analysis. An aliquot from the blank control, test concentrations, and abiotic control were sampled at the end of the exposure (day 3) and submitted for analysis. Back-up solutions for each sample also were provided. The day 0 samples were analyzed on the day of receipt. The day 3 samples were placed in refrigerator on the day of receipt (February 7, 2003) and were analyzed three days later (February 10, 2003). Aliquots of all test solutions were transferred to autosampler vials for analysis by HPLC. The nominal 50 mg/L and 100 mg/L samples were diluted 1:2 and 1:4 with AAP nutrient medium, respectively, before analysis.

3. Instrumentation and Conditions

Instrument:	Hewlett Packard Model 1100 HPLC
Column:	Zorbax RX-C18, 2.1 x 150 mm, 5 µm particle size
Mobile Phase:	70% Water 30% Acetonitrile
Flow Rate:	0.3 mL/min
Column Temperature:	35°C
Injection Volume:	100 µL
Detector:	UV absorbance at 210 nm
Run Time:	10.0 minutes

4. Quantitation

A primary stock solution of the reference compound, DMA (purity 98.8%), was made by dissolving 10.3 mg of the standard in 10 mL of acetonitrile. Calibration standards were prepared on each test day by dilution of the primary stock with AAP nutrient medium at concentrations ranging from 5.1 to 51 mg/L. Duplicate injections of calibration solutions were made and peak heights were determined by electronic integration.

The calibration curves were generated by regression analysis of the peak height data obtained from chromatographic analysis of the calibration solutions. Data for test solutions was fitted to the calibration curve to determine the concentrations of DMA. The limit of detection (LOD) and limit of quantitation (LOQ) were determined by calculating the average noise level in chromatograms of blank control samples and comparing them to the signal of a calibration standard of known concentration. Two chromatograms were examined for noise-related peaks near the retention time of the analyte. The LOD was calculated as 3 times the concentration

equivalent of the mean noise level. The LOQ was calculated as 10 times the concentration equivalent of the mean noise level.

RESULTS AND DISCUSSION

A. Environmental Conditions - Definitive and Recovery Tests (Tables 1-2)

- For the definitive test, the pH measurements of the test solutions ranged from 7.32 to 7.98 at the 0-hour and from 7.47 to 8.41 at the 72-hour interval.
- For the definitive test, light intensity for the areas used in the chamber ranged from 6460 to 8120 lumens/m² (= lux). The mean light intensity was 7168 lumens/m².
- For the definitive test, the shaking speed was 99 revolutions per min (rpm).
- For the definitive test, the temperature from the digital readout on the temperature recorder was 24.6°C for the duration of the study.

B. Test Substance Stability and Analytical Verification (Table 3, Figures 1-4)

1. Chromatographic Results

DMA eluted as a well-resolved peak with a retention time of approximately 4.4 minutes. Figure 1 shows a representative calibration curve obtained for DMA. A chromatogram of a typical standard is shown in Figure 2. Figures 3 and 4 show chromatograms of a blank control solution and test concentration solution, respectively.

The LOD and LOQ were determined to be 0.29 mg/L and 0.98 mg/L, respectively.

2. Test Solution Results

The mean, measured concentrations of DMA in the day 0 working stock (= abiotic control, = nominal 100 mg/L) solutions were 98 mg/L. This represents 98% recovery of the active ingredient. The mean, measured concentrations of DMA in the day 0 nominal 6.25, 12.5, 25, and 50 mg/L test concentration solutions were 5.5, 12, 25, and 49 mg/L, respectively. This represents 88, 96, 100, and 98% recovery of the active ingredient, respectively. These data indicated the stock and test concentration solutions were prepared at the desired concentrations. After 3 days, DMA was not detected in the 6.25, 12.5, and 25 mg/L test concentration solutions. The mean, measured concentrations of DMA in the 50 and 100 mg/L test concentrations were 12 and 44 mg/L, respectively. However, after 3 days, the mean, measured concentration of DMA in the abiotic control was 99 mg/L. The blank control solutions contained no detectable concentrations of DMA on both day 0 and day 3.

DMA was determined not to be stable in the presence of algae over the course of the definitive test as evidenced by the analytical recoveries obtained from the day 0 and day 3 test

concentrations solutions. However, DMA was stable over the course of the definitive test without algae as evidenced by the analytical recoveries obtained from the day 0 and day 3 abiotic control solution.

C. *Selenastrum capricornutum* Growth
(Tables 4-7, Appendices C-E)

1. Definitive Test

The organisms were exposed for 72 hours without test medium renewal. The effects were expressed in terms of percent inhibition in growth based on healthy cell count, area under the growth curve, and growth rate relative to the blank control for the 72-hour interval of the test. The 72-hour results are as follows:

Healthy Cell Count

EC ₅₀	> 100 mg DMA/L
NOEC	12.5 mg DMA/L

Area Under the Growth Curve

EC ₅₀	> 100 mg DMA/L
NOEC	50 mg DMA/L

Growth Rate

EC ₅₀	> 100 mg DMA/L
NOEC	12.5 mg DMA/L

The reductions in healthy cell count, area under the growth curve, and growth rate indicate a dose-dependent response with increasing concentrations of the test substance.

2. Recovery Test

None of the definitive test concentrations exhibited a 50% or greater growth inhibition based on healthy cell count relative to the blank control. Therefore, a recovery test was not performed.

CONCLUSIONS

The reductions in healthy cell count, area under the growth curve, and growth for *Selenastrum capricornutum* at 72 hours (3 days) indicate a dose-dependent response for increasing concentrations of the test substance, DMA.

The most sensitive parameters were healthy cell count and growth rate each with an EC₅₀ of > 100 mg/L and a NOEC of 12.5 mg/L.

RECORDS AND SAMPLE STORAGE

All data and records for analytical characterizations conducted by the sponsor will be archived by the sponsor. Laboratory-specific or site-specific raw data, such as personnel files and equipment records will be retained by the facility where the work was done.

Specimens (if applicable), raw data, and the final report will be retained at Haskell Laboratory, Newark, Delaware, or at Iron Mountain Records Management, Wilmington, Delaware (formerly known as the E.I. du Pont de Nemours and Company Records Management Center).

REFERENCES

1. DuPont Haskell Laboratory (2003). Solubility of H-24301 in AAP Nutrient Medium. Unpublished report, HA-2003-020.
2. Miller, W.E.; Greene, J.C.; Shiroyama, T. The *Selenastrum capricornutum* Printz Algal Assay Bottle Test; U.S. Environmental Protection Agency; U.S. Government Printing Office: Washington, DC, 1978; EPA-600/9-78-018.
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5. Tamhane, A.C., Dunnett, C.W., Green J.W., and Wetherington, J.D. (2001). Multiple test procedures for identifying maximum safe dose. *J Amer. Statist. Assoc.* **96**, 835-843.

TABLES

TABLE 1
pH MEASUREMENTS OF TEST SOLUTIONS

Nominal DMA Concentration	pH	
	Exposure Initiated: 04-Feb-2003 0-Hour (Day 0)	Exposure Ended: 07-Feb-2003 72-Hour (Day 3)
Blank Control	7.47	7.47
6.25 mg/L	7.49	8.41
12.5 mg/L	7.47	8.40
25 mg/L	7.51	8.34
50 mg/L	7.32	8.35
100 mg/L	7.98	8.13
Abiotic Control (100 mg/L/L)	7.98	7.51

TABLE 2
CHAMBER LIGHT INTENSITY, SHAKING SPEED, AND TEMPERATURE RANGE

Test	Mean Light Intensity at Test Initiation (lumens/m ² = lux)	Oscillations (rpms)	Temperature (°C)
Definitive	7168	99	24.6

TABLE 3
MEASURED CONCENTRATIONS OF DMA IN TEST SOLUTIONS

Sample Description ^a	Nominal DMA Concentration ^b (mg/L)	Measured DMA Concentration (mg/L)	
		Day 0	Day 3
Blank Control	0	ND ^c	ND
Working Stock (100 mg/L)	100	98	NS ^d
6.25 mg/L	6.25	5.5	ND
12.5 mg/L	12.5	12	ND
25 mg/L	25	25	ND
50 mg/L	50	49	12
100 mg/L	100	98 ^e	44
Abiotic Control (100 mg/L)	100	98 ^e	99

a Defined in terms of DMA/liter nutrient medium.

b DMA was 98.8% pure by analysis.

c ND stands for not detected. The limit of detection was calculated as 0.29 mg/L.

d NS stands for not submitted.

e No separate analysis was conducted since the working stock, the day 0 nominal 100 mg/L test concentration, and the day 0 abiotic control were aliquots of the same solution. Therefore, the measured concentration obtained for the working stock can be used for the 100 mg/L test concentration and the abiotic control.

TABLE 4
HEALTHY CELL COUNT DATA SUMMARY - DEFINITIVE TEST

Total Nominal Formaul Concentration	Rep.	Exposure Initiated:		Exposure Ended:	
		Day 0: 04 February 2003		Day 3: 07 February 2003	
		Healthy Cell Count (cells/mL) by Exposure Period			
		0-Hour	24-Hour	48-Hour	72-Hour
Blank Control	1	10,000	180,000	770,000	4,030,000
	2	10,000	250,000	1,130,000	4,050,000
	3	10,000	170,000	680,000	3,850,000
Mean		10,000	200,000	860,000	3,976,667
Standard Error		0.00	25,166.11	137,477.27	63,595.95
Std. Dev.		0.00	43,588.99	238,117.62	110,151.41
Variance		0.00E+00	1.90E+09	5.67E+10	1.21E+10
6.25 mg/L	1	10,000	150,000	920,000	4,380,000
	2	10,000	100,000	640,000	4,360,000
	3	10,000	120,000	600,000	4,600,000
Mean		10,000	123,333	720,000	4,446,667
Standard Error		0.00	14,529.66	100,664.46	76,883.75
Std. Dev.		0.00	25,166.11	174,355.96	133,166.56
Variance		0.00E+00	6.33E+08	3.04E+10	1.77E+10
% Inhibition		0.00	38.33	16.28	-11.82
12.5 mg/L	1	10,000	190,000	870,000	4,120,000
	2	10,000	170,000	930,000	4,260,000
	3	10,000	190,000	670,000	3,990,000
Mean		10,000	183,333	823,333	4,123,333
Standard Error		0.00	6,666.67	78,598.84	77,960.10
Std. Dev.		0.00	11,547.01	136,137.19	135,030.86
Variance		0.00E+00	1.33E+08	1.85E+10	1.82E+10
% Inhibition		0.00	8.33	4.26	-3.69
25 mg/L	1	10,000	280,000	1,070,000	3,230,000
	2	10,000	160,000	750,000	3,390,000
	3	10,000	160,000	620,000	3,380,000
Mean		10,000	200,000	813,333	3,333,333
Standard Error		0.00	40,000.00	133,707.81	51,747.25
Std. Dev.		0.00	69,282.03	231,588.72	89,628.86
Variance		0.00E+00	4.80E+09	5.36E+10	8.03E+09
% Inhibition		0.00	0.00	5.43	16.18
50 mg/L	1	10,000	230,000	800,000	3,400,000
	2	10,000	120,000	590,000	3,280,000
	3	10,000	150,000	930,000	4,020,000
Mean		10,000	166,667	773,333	3,566,667
Standard Error		0.00	32,829.53	99,051.05	229,298.45
Std. Dev.		0.00	56,862.41	171,561.46	397,156.56
Variance		0.00E+00	3.23E+09	2.94E+10	1.58E+11
% Inhibition		0.00	16.67	10.08	10.31
100 mg/L	1	10,000	150,000	710,000	3,480,000
	2	10,000	140,000	330,000	3,100,000
	3	10,000	160,000	640,000	3,560,000
Mean		10,000	150,000	560,000	3,380,000
Standard Error		0.00	5,773.50	116,761.87	141,891.98
Std. Dev.		0.00	10,000.00	202,237.48	245,764.11
Variance		0.00E+00	1.00E+08	4.09E+10	6.04E+10
% Inhibition		0.00	25.00	34.88	15.00

TABLE 5
AREA UNDER THE GROWTH CURVE DATA SUMMARY - DEFINITIVE TEST

Total Nominal Formaul Concentration	Rep.	Exposure Initiated:		Exposure Ended:	
		Day 0: 04 February 2003		Day 3: 07 February 2003	
		Area Under the Growth Curve Based on			
		Healthy Cell Count (cells/mL) by Exposure Period			
		0-24 Hour	0-48 Hour	0-72 Hour	
Blank Control	1	2,040,000	13,200,000	70,560,000	
	2	2,880,000	19,200,000	81,120,000	
	3	1,920,000	11,880,000	66,000,000	
Mean		2,280,000	14,760,000	72,560,000	
Standard Error		301,993.38	2,252,465.32	4,477,856.63	
Std. Dev.		523,067.87	3,901,384.37	7,755,875.19	
Variance		2.74E+11	1.52E+13	6.02E+13	
6.25 mg/L	1	1,680,000	14,280,000	77,640,000	
	2	1,080,000	9,720,000	69,480,000	
	3	1,320,000	9,720,000	71,880,000	
Mean		1,360,000	11,240,000	73,000,000	
Standard Error		174,355.96	1,520,000.00	2,421,239.35	
Std. Dev.		301,993.38	2,632,717.23	4,193,709.58	
Variance		9.12E+10	6.93E+12	1.76E+13	
% Inhibition		40.35	23.85	-0.61	
12.5 mg/L	1	2,160,000	14,640,000	74,280,000	
	2	1,920,000	14,880,000	76,920,000	
	3	2,160,000	12,240,000	67,920,000	
Mean		2,080,000	13,920,000	73,040,000	
Standard Error		80,000.00	842,852.30	2,671,029.76	
Std. Dev.		138,564.06	1,459,863.01	4,626,359.26	
Variance		1.92E+10	2.13E+12	2.14E+13	
% Inhibition		8.77	5.69	-0.66	
25 mg/L	1	3,240,000	19,200,000	70,560,000	
	2	1,800,000	12,480,000	61,920,000	
	3	1,800,000	10,920,000	58,680,000	
Mean		2,280,000	14,200,000	63,720,000	
Standard Error		480,000.00	2,540,236.21	3,545,588.81	
Std. Dev.		831,384.39	4,399,818.18	6,141,139.96	
Variance		6.91E+11	1.94E+13	3.77E+13	
% Inhibition		0.00	3.79	12.18	
50 mg/L	1	2,640,000	14,760,000	64,920,000	
	2	1,320,000	9,600,000	55,800,000	
	3	1,680,000	14,400,000	73,560,000	
Mean		1,880,000	12,920,000	64,760,000	
Standard Error		393,954.31	1,663,249.83	5,127,494.51	
Std. Dev.		682,348.88	2,880,833.21	8,881,081.02	
Variance		4.66E+11	8.30E+12	7.89E+13	
% Inhibition		17.54	12.47	10.75	
100 mg/L	1	1,680,000	11,760,000	61,800,000	
	2	1,560,000	6,960,000	47,880,000	
	3	1,800,000	11,160,000	61,320,000	
Mean		1,680,000	9,960,000	57,000,000	
Standard Error		69,282.03	1,509,966.89	4,562,104.78	
Std. Dev.		120,000.00	2,615,339.37	7,901,797.26	
Variance		1.44E+10	6.84E+12	6.24E+13	
% Inhibition		26.32	32.52	21.44	

TABLE 6
GROWTH RATE DATA SUMMARY - DEFINITIVE TEST

Total Nominal Formaul Concentration		Exposure Initiated:		Exposure Ended:	
		Day 0: 04 February 2003		Day 3: 07 February 2003	
		Growth Rate Based on			
		Healthy Cell Count (cells/mL) by Exposure Period			
Rep.		0-24 Hour	0-48 Hour	0-72 Hour	
Blank Control	1	0.1204	0.0905	0.0833	
	2	0.1341	0.0985	0.0834	
	3	0.1181	0.0879	0.0827	
Mean		0.1242	0.0923	0.0831	
Standard Error		0.0050	0.0032	0.0002	
Std. Dev.		0.0087	0.0055	0.0004	
Variance		7.48E-05	3.05E-05	1.43E-07	
6.25 mg/L	1	0.1128	0.0942	0.0845	
	2	0.0959	0.0866	0.0844	
	3	0.1035	0.0853	0.0852	
Mean		0.1041	0.0887	0.0847	
Standard Error		0.0049	0.0028	0.0003	
Std. Dev.		0.0085	0.0048	0.0004	
Variance		7.16E-05	2.31E-05	1.90E-07	
% Inhibition		16.21	3.90	-1.88	
12.5 mg/L	1	0.1227	0.0930	0.0836	
	2	0.1181	0.0944	0.0841	
	3	0.1227	0.0876	0.0832	
Mean		0.1212	0.0917	0.0836	
Standard Error		0.0015	0.0021	0.0003	
Std. Dev.		0.0027	0.0036	0.0005	
Variance		7.05E-06	1.29E-05	2.03E-07	
% Inhibition		2.44	0.69	-0.60	
25 mg/L	1	0.1388	0.0974	0.0802	
	2	0.1155	0.0899	0.0809	
	3	0.1155	0.0860	0.0809	
Mean		0.1233	0.0911	0.0807	
Standard Error		0.0078	0.0033	0.0002	
Std. Dev.		0.0135	0.0058	0.0004	
Variance		1.81E-04	3.36E-05	1.63E-07	
% Inhibition		0.75	1.30	2.97	
50 mg/L	1	0.1306	0.0913	0.0810	
	2	0.1035	0.0849	0.0805	
	3	0.1128	0.0944	0.0833	
Mean		0.1156	0.0902	0.0816	
Standard Error		0.0080	0.0028	0.0009	
Std. Dev.		0.0138	0.0048	0.0015	
Variance		1.90E-04	2.35E-05	2.23E-06	
% Inhibition		6.90	2.28	1.84	
100 mg/L	1	0.1128	0.0888	0.0813	
	2	0.1100	0.0728	0.0797	
	3	0.1155	0.0866	0.0816	
Mean		0.1128	0.0827	0.0809	
Standard Error		0.0016	0.0050	0.0006	
Std. Dev.		0.0028	0.0087	0.0010	
Variance		7.56E-06	7.52E-05	1.04E-06	
% Inhibition		9.21	10.36	2.73	

TABLE 7
72-HOUR (DAY 3) STATISTICAL ANALYSES SUMMARY - DEFINITIVE TEST

	Nominal DMA Concentration	Mean Response	Standard Error	N	Test Concentration Response (mg/L)	
					Level	Value
Healthy Cell Count	Blank Control	3,976,667	63,596			
	6.25 mg/L	4,446,667	76,884	3	EC ₅₀ ^a	> 100
	12.5 mg/L	4,123,333	77,960	3		
	25 mg/L	3,333,333	51,747	3	NOEC	12.5
	50 mg/L	3,566,667	229,298	3		
	100 mg/L	3,380,000	141,892	3		
Area Under the Growth Curve	Blank Control	72,560,000	4,477,857			
	6.25 mg/L	73,000,000	2,421,239	3	EC ₅₀	> 100
	12.5 mg/L	73,040,000	2,671,030	3		
	25 mg/L	63,720,000	3,545,589	3	NOEC	50
	50 mg/L	64,760,000	5,127,495	3		
	100 mg/L	57,000,000	4,562,105	3		
Growth Rate	Blank Control	0.0831	0.0002			
	6.25 mg/L	0.0847	0.0003	3	EC ₅₀	> 100
	12.5 mg/L	0.0836	0.0003	3		
	25 mg/L	0.0807	0.0002	3	NOEC	12.5
	50 mg/L	0.0816	0.0009	3		
	100 mg/L	0.0809	0.0006	3		

a EC₅₀ was calculated using Probit analysis.

FIGURES

FIGURE 1
REPRESENTATIVE ANALYTICAL CALIBRATION STANDARD CURVE FOR DMA

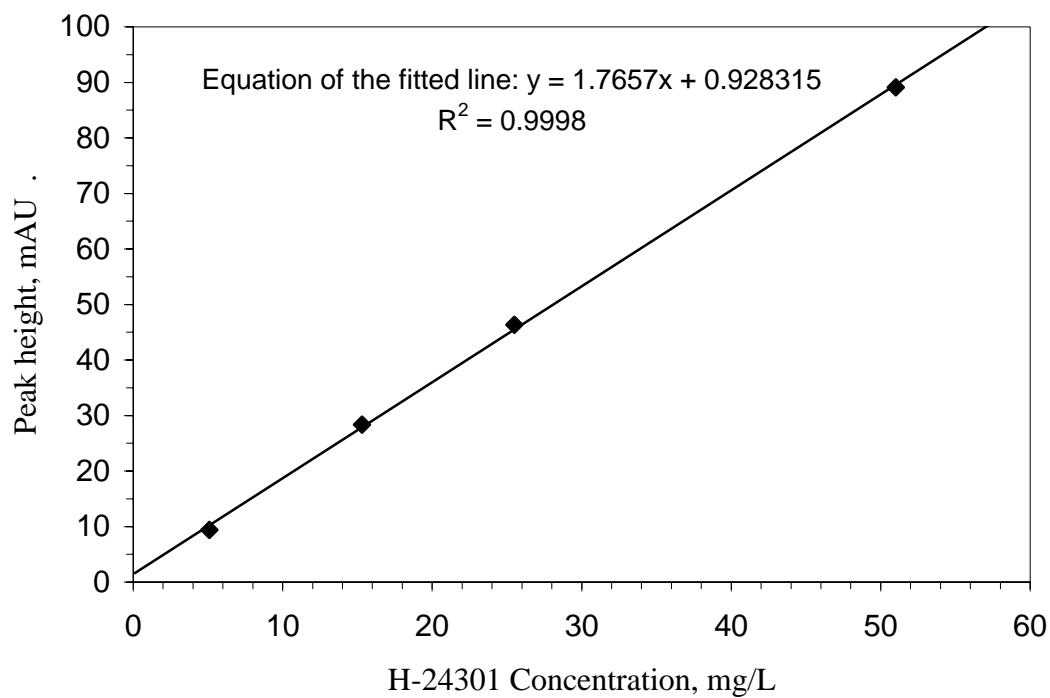
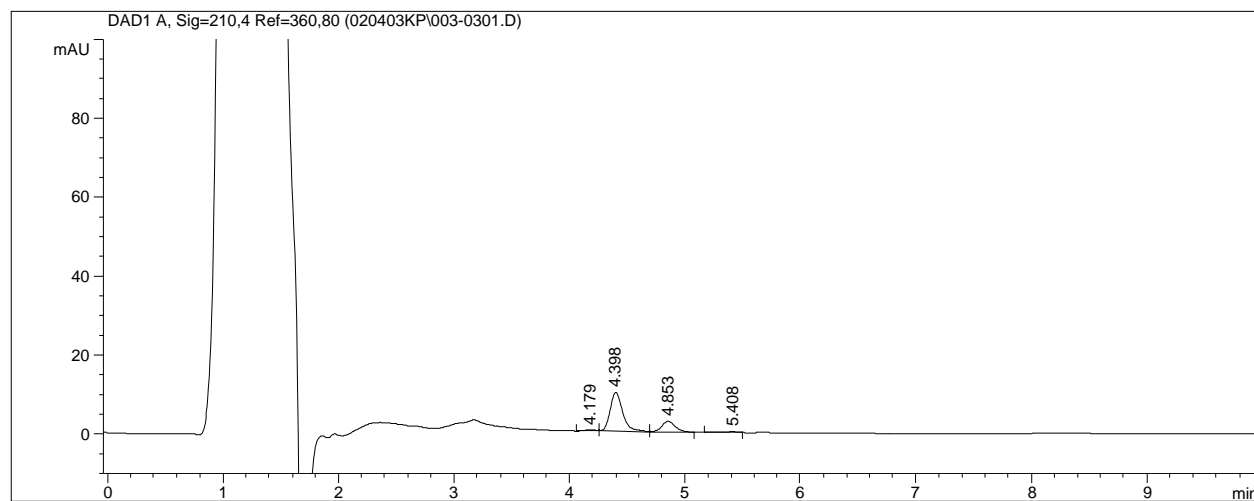
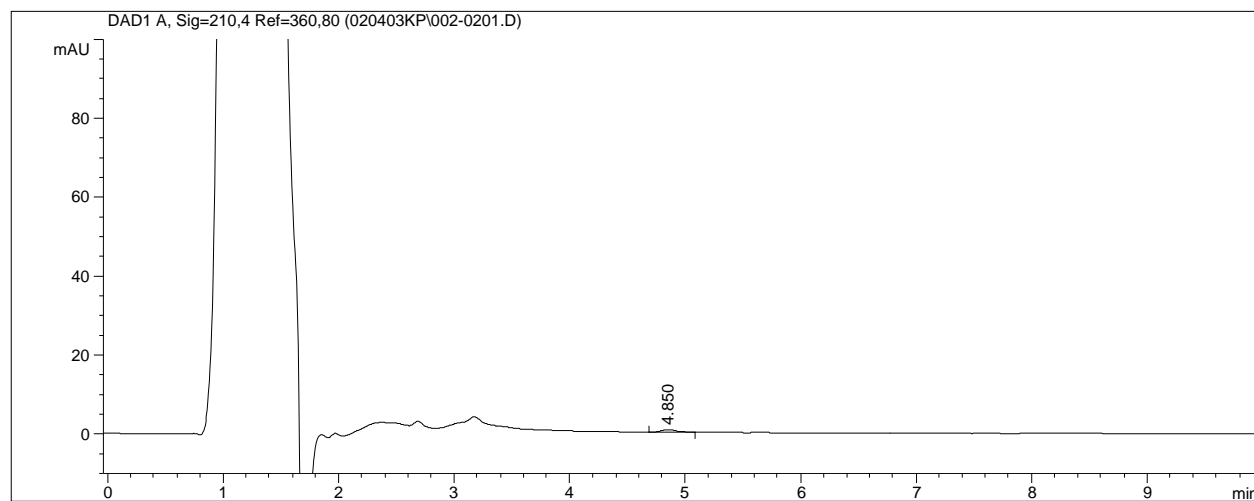


FIGURE 2
REPRESENTATIVE CHROMATOGRAM OF A
CALIBRATION STANDARD SOLUTION



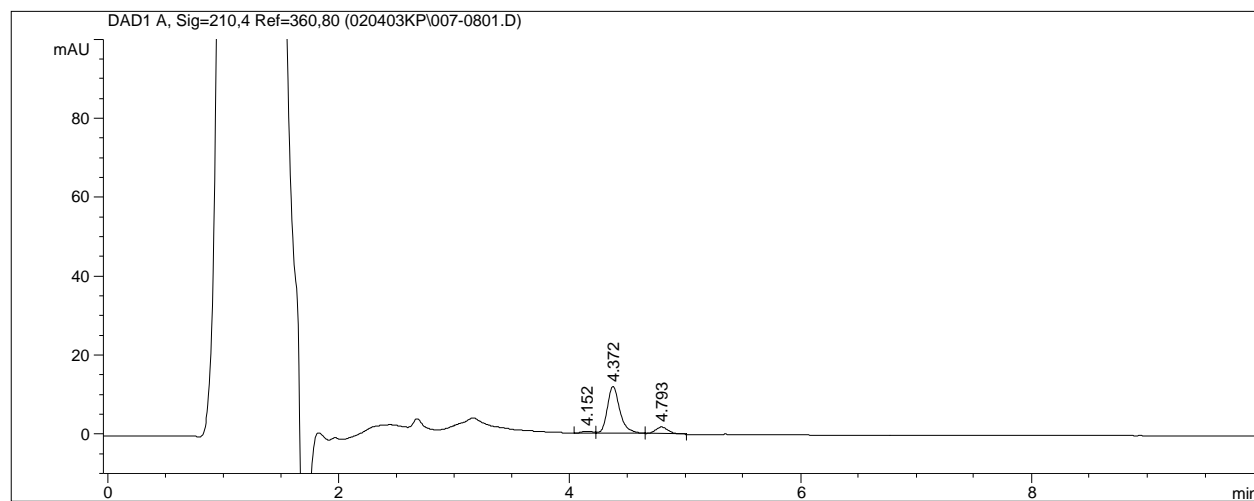
DMA elutes at a retention time of approximately 4.4 minutes. Calibration standard solution contains DMA at a concentration of 5.1 mg/L.

FIGURE 3
REPRESENTATIVE CHROMATOGRAM OF
THE BLANK CONTROL SOLUTION



DMA would elute at a retention time of approximately 4.4 minutes.

FIGURE 4
REPRESENTATIVE CHROMATOGRAM OF A
TEST CONCENTRATION SOLUTION



DMA elutes at a retention time of approximately 4.4 minutes. Test solution sample contains DMA at a nominal concentration of 6.25 mg/L.

APPENDICES

APPENDIX A

AAP Nutrient Medium Constituents

AAP NUTRIENT MEDIUM CONSTITUENTS

Stock Solutions ^a		Final Prepared Medium	
Component	Concentration (g/L)	Component	Concentration (mg/L)
Macronutrient Stock #1			
NaNO ₃	25.500	Na	11.001
		N	4.200
Macronutrient Stock #2			
NaHCO ₃	15.000	C	2.143
		K	0.469
Macronutrient Stock #3			
K ₂ HPO ₄	1.044	P	0.186
		Mg	2.904 ^b
Macronutrient Stock #4			
MgSO ₄ •7H ₂ O	14.700	Ca	1.202
		S	1.911
Macronutrient Stock #5			
MgCl ₂ •6H ₂ O	12.164		
Macronutrient Stock #6			
CaCl ₂ •2H ₂ O	4.410		
Stock Solutions ^a		Final Prepared Medium	
Component	Concentration (mg/L)	Component	Concentration (µg/L)
Micronutrient Stock			
H ₃ BO ₃	185.520	B	32.460
MnCl ₂ •4 H ₂ O	415.610	Mn	115.374
ZnCl ₂	3.271	Zn	1.570
CoCl ₂ •6 H ₂ O	1.428	Co	0.354
CuCl ₂ •2H ₂ O	0.012	Cu	0.004
Na ₂ MoO ₄ •2 H ₂ O	7.260	Mo	2.878
FeCl ₃ •6 H ₂ O	160.000	Fe	33.051
Na ₂ EDTA•2 H ₂ O	300.000		

a As reported in Reference 2.

b Includes magnesium from both magnesium sulfate and magnesium chloride.

APPENDIX B

72-Hour Healthy Cell Count Statistics for the Definitive Test

72-HOUR HEALTHY CELL COUNT STATISTICS FOR THE DEFINITIVE TEST

```

Enter k, lambda and alpha
k = 5      lambda = .50      alpha = .050

Enter n_0, n_1, ..., n_k
Sample sizes: 3. 3. 3. 3. 3. 3.

Enter ybar_0, ybar_1, ... , ybar_k
Means:      725.60      730.00      730.40      637.20      647.60      570.00

Enter sigma^2, and d.f. (d.f. = 0 for inf.)
sigma^2 =    4636.16    d.f. = 12

Contrast coefficients for testing dose 1:-

      j      0      1      2      3      4      5
1      -5.      1.      1.      1.      1.      1.
2      -4.      1.      1.      1.      1.      0.
3      -3.      1.      1.      1.      0.      0.
4      -2.      1.      1.      0.      0.      0.
5      -1.      1.      0.      0.      0.      0.
      362.80  730.00  730.40  637.20  647.60  570.00

      contrast      s.e.      t
      1501.20      131.85      11.39
      1294.00      111.19      11.64
      1009.20      90.07      11.20
      734.80      68.09      10.79
      367.20      43.95      8.35
t_max = 11.64  rhob = .7916  Adj. p-value = .000

Contrast coefficients for testing dose 2:-

      j      0      2      3      4      5
1      -4.      1.      1.      1.      1.
2      -3.      1.      1.      1.      0.
3      -2.      1.      1.      0.      0.
4      -1.      1.      0.      0.      0.
      362.80  730.40  637.20  647.60  570.00

      contrast      s.e.      t
      1134.00      111.19      10.20
      926.80      90.07      10.29
      642.00      68.09      9.43
      367.60      43.95      8.36
t_max = 10.29  rhob = .7857  Adj. p-value = .000

```

Contrast coefficients for testing dose 3:-

j	0	3	4	5
1	-3.	1.	1.	1.
2	-2.	1.	1.	0.
3	-1.	1.	0.	0.
	362.80	637.20	647.60	570.00
contrast	s.e.		t	
	766.40		8.51	
	559.20		8.21	
	274.40		6.24	
t_max =	8.51	rhob = .7799	Adj. p-value = .000	

Contrast coefficients for testing dose 4:-

j	0	4	5
1	-2.	1.	1.
2	-1.	1.	0.
	362.80	647.60	570.00
contrast	s.e.		t
	492.00		7.23
	284.80		6.48
t_max =	7.23	rhob = .7746	Adj. p-value = .000

Contrast coefficients for testing dose 5:-

j	0	5	
1	-1.	1.	
	362.80	570.00	
	contrast	s.e.	t
	207.20	43.95	4.71
t_max =	4.71	rhob = .0000	Adj. p-value = .000

Est. MAXSD = 5 (alpha = .050)

Thus the MAXSD is 100 mg/L, so that, with 95% confidence, the effect at dose 5 (100) is less than 50%.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 17:13 TUESDAY 25FEB03
ECOTOX MEASUREMENTS FROM DATASET AWR14398
GROUP STATISTICS FOR formula1 BY DOSE

dose	doseval (mg/L)	COUNT	MEAN	MEDIAN	STD_DEV	STD_ERR
1	0	3	39.7667	40.3	1.10151	0.63596
2	6.25	3	44.4667	43.8	1.33167	0.76884
3	12.5	3	41.2333	41.2	1.35031	0.77960
4	25	3	33.3333	33.8	0.89629	0.51747
5	50	3	35.6667	34.0	3.97157	2.29298
6	100	3	33.8000	34.8	2.45764	1.41892

SHAPIRO-WILK TEST OF NORMALITY OF formula1
AQUATIC SELENASTRUM: FULL DATA

OBS	STD	SKEW	KURT	SW_STAT	P_VALUE	SIGNIF
18	1.79629	0.57535	1.11942	0.95431	0.49649	

Outliers & Influential Observations

Obs	SELENASTRUM	dose	doseval	group	OBSER	Pred
1	3	5	50	V	40.2	35.6667
Obs	SE_PRED	L95M	U95M	Resid	LB	UB
1	1.23438	32.9772	38.3562	4.53333	-4.66667	4.4

LEVENE TEST FOR formula1 - FULL Model

Effect	DF	LEVENE	P_VALUE	SIGNIF
DOSE	5	0.51879	0.75765	

The data was found to be normally distributed with equal variances.
An analysis of variance will be performed.

DOSES 0, 6.25, 12.5, 25, 50, 100 mg/L

Obs	Class	Levels	Values
1	dose	6	1 2 3 4 5 6

OVERALL F-TESTS FOR ANOVA

Obs	Effect	Num DF	Den DF	FValue	ProbF
1	dose	5	12	13.16	0.0002

LSMEANS - DOSE MEANS ADJUSTED FOR THE EFFECTS OF SELENASTRUM

Obs	LEVEL	LSMEAN	SE	DDF
1	0	39.7667	1.23438	12
2	6.25	44.4667	1.23438	12
3	12.5	41.2333	1.23438	12
4	25	33.3333	1.23438	12
5	50	35.6667	1.23438	12
6	100	33.8000	1.23438	12

ESTIMATED DOSE EFFECTS & DUNNETT FOR Two-sided ALTERNATIVE
USING ALPHA=.05 FOR COMPARISONS TO CONTROL

Estimate	SIGNIF	Dunnett 2-sided p-value	Test Group Mean	N
DOSE TREND	**	0.00005	.	.
DOSE QUAD		0.36872	.	.
DOSE 2-1		0.07195	44.4667	3
DOSE 3-1		0.86499	41.2333	3
DOSE 4-1	*	0.01248	33.3333	3
DOSE 5-1		0.12893	35.6667	3
DOSE 6-1	*	0.02004	33.8000	3

KEY

ZC IS JONCKHEERE STATISTIC COMPUTED WITH TIE CORRECTION

ZCCF IS ZC WITH CONTINUITY CORRECTION FACTOR

PlUPCF IS P-VALUE FOR UPWARD TREND

PlDNCF IS P-VALUE FOR DOWNWARD TREND

P-VALUES ARE FOR TIE-CORRECTED TEST WITH CONTINUITY CORRECTION FACTOR

SIGNIF RESULTS ARE FOR A TWO-SIDED ALTERNATIVE HYPOTHESIS

Jonckheere Trend Test on Dose 0 and Lowest 5 Doses through 100 mg/L

JONC	ZC	ZCCF	PlUPCF	PlDNCF	SIGNIF
35	-2.501851	-2.463361	0.9931179	0.0055372	*

Jonckheere Trend Test on Dose 0 and Lowest 4 Doses through Dose 50 mg/L

JONC	ZC	ZCCF	PlUPCF	PlDNCF	SIGNIF
25	-2.025479	-1.974842	0.9758569	0.0189416	*

Jonckheere Trend Test on Dose 0 and Lowest 3 Doses through Dose 25 mg/L

JONC	ZC	ZCCF	PlUPCF	PlDNCF	SIGNIF
16	-1.563472	-1.492405	0.9322035	0.0510729	

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Jonckheere test results are included in the summary table. Group means should be examined to check for lack-of-fit to a linear trend before trend test results are accepted.

APPENDIX C

72-Hour Area Under the Growth Curve Statistics for the Definitive Test

72-HOUR AREA UNDER THE GROWTH CURVE STATISTICS FOR THE DEFINITIVE TEST

MAXSD Calculations for Formula2

Formulation 2 (Max. Safe Dose, or MAXSD):-

Enter k, lambda and alpha

k = 5 lambda = .50 alpha = .050

Enter n_0, n_1, ..., n_k

Sample sizes: 3. 3. 3. 3. 3.

Enter ybar_0, ybar_1, ..., ybar_k

Means: 39.77 44.47 41.23 33.33 35.67 33.80

Enter sigma^2, and d.f. (d.f. = 0 for inf.)

sigma^2 = 4.57 d.f. = 12

Contrast coefficients for testing dose 1:-

j	0	1	2	3	4	5
1	-5.	1.	1.	1.	1.	1.
2	-4.	1.	1.	1.	1.	0.
3	-3.	1.	1.	1.	0.	0.
4	-2.	1.	1.	0.	0.	0.
5	-1.	1.	0.	0.	0.	0.
	19.89	44.47	41.23	33.33	35.67	33.80

contrast	s.e.	t
89.07	4.14	21.52
75.16	3.49	21.53
59.38	2.83	21.00
45.93	2.14	21.49
24.59	1.38	17.82

t_max = 21.53 rhob = .7916 Adj. p-value = .000

Contrast coefficients for testing dose 2:-

j	0	2	3	4	5
1	-4.	1.	1.	1.	1.
2	-3.	1.	1.	1.	0.
3	-2.	1.	1.	0.	0.
4	-1.	1.	0.	0.	0.
	19.89	41.23	33.33	35.67	33.80

contrast	s.e.	t
64.49	3.49	18.47
50.58	2.83	17.88
34.79	2.14	16.27
21.34	1.38	15.47

t_max = 18.47 rhob = .7857 Adj. p-value = .000

Contrast coefficients for testing dose 3:-

j	0	3	4	5
1	-3.	1.	1.	1.
2	-2.	1.	1.	0.
3	-1.	1.	0.	0.
	19.89	33.33	35.67	33.80
contrast	s.e.	t		
43.15	2.83	15.26		
29.23	2.14	13.67		
13.45	1.38	9.74		
t_max = 15.26	rhob = .7799	Adj. p-value = .000		

Contrast coefficients for testing dose 4:-

j	0	4	5
1	-2.	1.	1.
2	-1.	1.	0.
	19.89	35.67	33.80
contrast	s.e.	t	
	29.70	2.14	13.89
	15.78	1.38	11.44
t_max = 13.89	rhob = .7746	Adj. p-value = .000	

Contrast coefficients for testing dose 5:-

j	0	5
1	-1.	1.
	19.89	33.80
contrast	s.e.	t
13.91	1.38	10.08
t_max = 10.08	rhob = .0000	Adj. p-value = .000

Est. MAXSD = 5 (alpha = .050)
Thus the MAXSD is 100 mg/L, so that, with 95% confidence, the effect at dose 5 (100) is less than 50%.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 17:13 TUESDAY 25FEB03
ECOTOX MEASUREMENTS FROM DATASET AWR14398
GROUP STATISTICS FOR formula2 BY DOSE

dose	doseval (mg/L)	COUNT	MEAN	MEDIAN	STD_DEV	STD_ERR
1	0	3	725.6	705.6	77.5588	44.7786
2	6.25	3	730.0	718.8	41.9371	24.2124
3	12.5	3	730.4	742.8	46.2636	26.7103
4	25	3	637.2	619.2	61.4114	35.4559
5	50	3	647.6	649.2	88.8108	51.2749
6	100	3	570.0	613.2	79.0180	45.6210

SHAPIRO-WILK TEST OF NORMALITY OF formula2

OBS	STD	SKEW	KURT	SW_STAT	P_VALUE	SIGNIF
18	57.2065	-0.035747	-1.11923	0.95296	0.47326	

LEVENE TEST FOR formula2 - FULL Model

Effect	DF	LEVENE	P_VALUE	SIGNIF
DOSE	5	0.18087	0.96452	

The data was found to be normally distributed with equal variances.
An analysis of variance will be performed.

DOSES 0, 6.25, 12.5, 25, 50, 100 mg/L

Obs	Class	Levels	Values
1	dose	6	1 2 3 4 5 6

OVERALL F-TESTS FOR ANOVA

Obs	Effect	Num DF	Den DF	FValue	ProbF
1	dose	5	12	2.83	0.0651

LSMEANS - DOSE MEANS ADJUSTED FOR THE EFFECTS OF SELENASTRUM

Obs	LEVEL	LSMEAN	SE	DDF
1	0	725.6	39.3114	12
2	6.25	730.0	39.3114	12
3	12.5	730.4	39.3114	12
4	25	637.2	39.3114	12
5	50	647.6	39.3114	12
6	100	570.0	39.3114	12

ESTIMATED DOSE EFFECTS & DUNNETT FOR Two-sided ALTERNATIVE
USING ALPHA=.05 FOR COMPARISONS TO CONTROL

Estimate	SIGNIF	Dunnett 2-sided p-value	Test Group Mean	N
DOSE TREND	**	0.00527	.	.
DOSE QUAD		0.32471	.	.
DOSE 2-1		1.00000	730.0	3
DOSE 3-1		1.00000	730.4	3
DOSE 4-1		0.40793	637.2	3
DOSE 5-1		0.51668	647.6	3
DOSE 6-1		0.05981	570.0	3

KEY

ZC IS JONCKHEERE STATISTIC COMPUTED WITH TIE CORRECTION

ZCCF IS ZC WITH CONTINUITY CORRECTION FACTOR

P1UPCF IS P-VALUE FOR UPWARD TREND

P1DNCF IS P-VALUE FOR DOWNWARD TREND

P-VALUES ARE FOR TIE-CORRECTED TEST WITH CONTINUITY CORRECTION FACTOR

SIGNIF RESULTS ARE FOR A TWO-SIDED ALTERNATIVE HYPOTHESIS

Jonckheere Trend Test on Dose 0 and Lowest 5 Doses through 100 mg/L

JONC	ZC	ZCCF	P1UPCF	P1DNCF	SIGNIF
31.5	-2.773094	-2.734579	0.996877	0.0024647	**

Jonckheere Trend Test on Dose 0 and Lowest 4 Doses through Dose 50 mg/L

JONC	ZC	ZCCF	P1UPCF	P1DNCF	SIGNIF
27.5	-1.774245	-1.723552	0.9576056	0.0340052	

Jonckheere test results are included in the summary table. Group means should be examined to check for lack-of-fit to a linear trend before trend test results are accepted.

APPENDIX D

72-Hour Hour Growth Rate Statistics for the Definitive Test

72-HOUR GROWTH RATE STATISTICS FOR THE DEFINITIVE TEST

Enter k, lambda and alpha

k = 5 lambda = .50 alpha = .050

Enter n_0, n_1, ..., n_k

Sample sizes: 3. 3. 3. 3. 3. 3.

Enter ybar_0, ybar_1, ... , ybar_k

Means: .08 .08 .08 .08 .08 .08

Enter sigma^2, and d.f. (d.f. = 0 for inf.)

sigma^2 = .00 d.f. = 12

Contrast coefficients for testing dose 1:-

j	0	1	2	3	4	5
1	-5.	1.	1.	1.	1.	1.
2	-4.	1.	1.	1.	1.	0.
3	-3.	1.	1.	1.	0.	0.
4	-2.	1.	1.	0.	0.	0.
5	-1.	1.	0.	0.	0.	0.
	.04	.08	.08	.08	.08	.08

contrast	s.e.	t
.20	.05	4.09
.16	.04	3.91
.12	.03	3.65
.09	.03	3.31
.04	.02	2.60

t_max = 4.09 rhob = .7916 Adj. p-value = .002

Contrast coefficients for testing dose 2:-

j	0	2	3	4	5
1	-4.	1.	1.	1.	1.
2	-3.	1.	1.	1.	0.
3	-2.	1.	1.	0.	0.
4	-1.	1.	0.	0.	0.
	.04	.08	.08	.08	.08

contrast	s.e.	t
.16	.04	3.82
.12	.03	3.56
.08	.03	3.15
.04	.02	2.53

t_max = 3.82 rhob = .7857 Adj. p-value = .003

Contrast coefficients for testing dose 3:-

j	0	3	4	5
1	-3.	1.	1.	1.
2	-2.	1.	1.	0.
3	-1.	1.	0.	0.
	.04	.08	.08	.08
contrast	s.e.		t	
	.12	.03	3.48	
	.08	.03	3.08	
	.04	.02	2.35	
t_max =	3.48	rhob = .7799	Adj. p-value = .005	

Contrast coefficients for testing dose 4:-

j	0	4	5
1	-2.	1.	1.
2	-1.	1.	0.
	.04	.08	.08
contrast		s.e.	t
	.08	.03	3.08
	.04	.02	2.41
t_max =	3.08	rhob = .7746	Adj. p-value = .008

Contrast coefficients for testing dose 5:-

j	0	5	
1	-1.	1.	
	.04	.08	
	contrast	s.e.	t
	.04	.02	2.37
t_max =	2.37	rhob = .0000	Adj. p-value = .018

Est. MAXSD = 5 (alpha = .050)

Thus the MAXSD is 100 mg/L, so that, with 95% confidence, the effect at dose 5 (100) is less than 50%.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 17:13 TUESDAY 25FEB03
ECOTOX MEASUREMENTS FROM DATASET AWR14398
GROUP STATISTICS FOR G_Rate BY DOSE

dose	doseval (mg/L)	COUNT	MEAN	MEDIAN	STD_DEV	STD_ERR
1	0	3	0.083133	0.0833	.000378594	.000218581
2	6.25	3	0.084700	0.0845	.000435890	.000251661
3	12.5	3	0.083633	0.0836	.000450925	.000260342
4	25	3	0.080667	0.0809	.000404145	.000233333
5	50	3	0.081600	0.0810	.001493318	.000862168
6	100	3	0.080867	0.0813	.001021437	.000589727

SHAPIRO-WILK TEST OF NORMALITY OF G_Rate

OBS	STD	SKEW	KURT	SW_STAT	P_VALUE	SIGNIF
18	.000683704	0.46175	1.13365	0.95716	0.54783	

LEVENE TEST FOR G_Rate - FULL Model

Effect	DF	LEVENE	P_VALUE	SIGNIF
DOSE	5	0.561	0.72823	

The data was found to be normally distributed with equal variances.
An analysis of variance will be performed.

DOSES 0, 6.25, 12.5, 25, 50, 100 mg/L

Obs	Class	Levels	Values
1	dose	6	1 2 3 4 5 6

OVERALL F-TESTS FOR ANOVA

Obs	Effect	Num DF	Den DF	FValue	ProbF
1	dose	5	12	12.08	0.0002

LSMEANS - DOSE MEANS ADJUSTED FOR THE EFFECTS OF SELENASTRUM

Obs	LEVEL	LSMEAN	SE	DDF
1	0	0.083133	.000469831	12
2	6.25	0.084700	.000469831	12
3	12.5	0.083633	.000469831	12
4	25	0.080667	.000469831	12
5	50	0.081600	.000469831	12
6	100	0.080867	.000469831	12

ESTIMATED DOSE EFFECTS & DUNNETT FOR Two-sided ALTERNATIVE
USING ALPHA=.05 FOR COMPARISONS TO CONTROL

Estimate	SIGNIF	Dunnett 2-sided p-value	Test Group Mean	N
DOSE TREND	**	0.00006	.	.
DOSE QUAD		0.43216	.	.
DOSE 2-1		0.12697	0.084700	3
DOSE 3-1		0.90606	0.083633	3
DOSE 4-1	*	0.01189	0.080667	3
DOSE 5-1		0.13797	0.081600	3
DOSE 6-1	*	0.02028	0.080867	3

KEY

ZC IS JONCKHEERE STATISTIC COMPUTED WITH TIE CORRECTION

ZCCF IS ZC WITH CONTINUITY CORRECTION FACTOR

P1UPCF IS P-VALUE FOR UPWARD TREND

P1DNCF IS P-VALUE FOR DOWNWARD TREND

P-VALUES ARE FOR TIE-CORRECTED TEST WITH CONTINUITY CORRECTION FACTOR

SIGNIF RESULTS ARE FOR A TWO-SIDED ALTERNATIVE HYPOTHESIS

Jonckheere Trend Test on Dose 0 and Lowest 5 Doses through 100 mg/L

JONC	ZC	ZCCF	P1UPCF	P1DNCF	SIGNIF
35.5	-2.466588	-2.428047	0.9924098	0.0061204	*

Jonckheere Trend Test on Dose 0 and Lowest 4 Doses through Dose 50 mg/L

JONC	ZC	ZCCF	P1UPCF	P1DNCF	SIGNIF
25.5	-1.979196	-1.928448	0.9731003	0.0211811	*

Jonckheere Trend Test on Dose 0 and Lowest 3 Doses through Dose 25 mg/L

JONC	ZC	ZCCF	P1UPCF	P1DNCF	SIGNIF
16	-1.566712	-1.495498	0.9326078	0.0507185	

Jonckheere test results are included in the summary table. Group means should be examined to check for lack-of-fit to a linear trend before trend test results are accepted.